PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A01N 63/02, C12N 15/32
C12P 21/02 // C12N 15/62

A1

(11) International Publication Number: **WO 91/01087**

(43) International Publication Date: 7 February 1991 (07.02.91)

(21) International Application Number: PCT/EP90/01145

(22) International Filing Date: 12 July 1990 (12.07.90)

(30) Priority data:
21243 A/89 20 July 1989 (20.07.89) IT

(71) Applicant *(for all designated States except US):* C.R.C. - COMPAGNIA DI RICERCA CHIMICA S.P.A. [IT/IT]; Via Pesenalat, 6, I-33048 San Giovanni al Natisone (IT).

(72) Inventors; and
(75) Inventors/Applicants *(for US only) :* GALIZZI , Alessandro [IT/IT]; Via Basilicata, 13, I-27100 Pavia (IT). ALBERTINI, Alessandra [IT/IT]; Via Goldoni, 7, I-27100 Pavia (IT). CARAMORI, Tiziana [IT/IT]; Strada Statale dei Giovi, 45, I-20082 Binasco (IT). DEGRASSI, Giuliano [IT/IT]; Via Brigata Macerata, 13, I-34077 Ronchi dei Legionari (IT). PERSIC, Lidija [YU/IT]; Via delle Case, 23/E, I-34011 Aurisina (IT).

(74) Agents: FORATTINI, A. et al.; Zini, Maranesi & C. S.r.l., Piazza Castello 1, I-20121 Milano (IT).

(81) Designated States: AT (European patent), AU, BE (European patent), BR, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), SU, US.

Published
With international search report.

(54) Title: NEW FUNCTIONAL BACILLUS THURINGIENSIS HYBRID GENES OBTAINED BY IN VIVO RECOMBINATION

(57) Abstract

A process for altering the target insect range (spectrum) of pesticidal toxins which comprises recombining *in vivo* the hypervariable regions of two genes encoding a pesticidal toxin and having enough residual homology as to be able to promote *in vivo* recombination. According to the present invention, truncated genes obtained from well known strains of *Bacillus thuringiensis* variety *kurstaki* and separated by an antibiotic resistant marker gene - or part of it - are cloned in a plasmid vector which is then introduced in a strain of *E. coli*. *In vivo* recombination between the hypervariable regions of the toxins genes reconstitutes an entire hybrid toxin gene. Polypeptides encoded by these new hybrid toxin genes have different biological activity and an altered target insect range as compared to their parental toxin.

* See back of page

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**ES**	Spain	**MC**	Monaco
AU	Australia	**FI**	Finland	**MG**	Madagascar
BB	Barbados	**FR**	France	**ML**	Mali
BE	Belgium	**GA**	Gabon	**MR**	Mauritania
BF	Burkina Fasso	**GB**	United Kingdom	**MW**	Malawi
BG	Bulgaria	**GR**	Greece	**NL**	Netherlands
BJ	Benin	**HU**	Hungary	**NO**	Norway
BR	Brazil	**IT**	Italy	**PL**	Poland
CA	Canada	**JP**	Japan	**RO**	Romania
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SN**	Senegal
CM	Cameroon	**LI**	Liechtenstein	**SU**	Soviet Union
DE	Germany	**LK**	Sri Lanka	**TD**	Chad
DK	Denmark	**LU**	Luxembourg	**TG**	Togo
				US	United States of America

NEW FUNCTIONAL BACILLUS THURINGIENSIS HYBRID GENES OBTAINED BY IN VIVO RECOMBINATION

Bacillus thuringiensis is a spore-forming bacterium which, upon sporulation, produces an insecticidal parasporal crystal. Many Bacillus thuringiensis subspecies have been isolated and the vast majority of the strains which have been tested have shown specific activity only against larvae of Lepidopteran insects such as Manduca sexta, Heliotis virescens and Trichoplusia ni. Of the Lepidopterans, not all are equally sensitive to Bacillus thuringiensis For example, Spodoptera species tend to be relatively insensitive.

The Lepidopteran specific Bacillus thuringiensis strains have been categorized according to flagellar serotype, crystal serotype as well as activity spectrum against various insects (Dulmage, 1981). Among the better studied varieties of Lepidopteran specific Bacillus thuringiensis are B.t. kurstaki HD1 which is the strain used in "Dipel", B.t. HD 73, B.t. dendrolimus, B.t. sotto, B.t. Berliner.

In recent years, new types of Bacillus thuringiensis with novel insecticidal specificities have been discovered. B.t. israelensis is toxic to larvae of several Dipteran species (mosquitoes and black flies), but not to Lepidopteran larvae (Goldberg and Margaht, 1977). More recently, two Coleopteran specific strains, B.t. tenebrionis and B.t. San Diego, which later were shown to be the same strain (Krieg et al., 1987), have been described (Krieg et

al., 1983, Krieg et al., 1984; Herrnstadt et al., 1986). These strains have shown activity against Colorado potato beetle and other Coleopteran pests.

The entomopathogenic activity of the Bacillus thuringiensis parasporal crystal is due to its composition: in the case of the Bacillus thuringiensis subspecies specifically active against Lepidopteran larvae, it is composed of 130 to 160 Kdal protoxin polypeptides. Different subspecies and often individual strains of the same subspecies produce endotoxins having a characteristic spectrum of insect toxicity (Whiteley and Schnepf, 1986; Andrews et al., 1987).

For many years Bacillus thuringiensis has served as the basis of successful biological insecticides. To produce these insecticides, Bacillus thuringiensis is fermented until spores and crystals are obtained. The mixture of spores and crystals is then formulated to allow effective application on crop plants..

Current, traditional Bacillus thuringiensis products are in fact an example of classical industrial microbiology. These products are created through such traditional microbiological practices as strain isolation and improvement, and fermentation optimization.

Two features of Bacillus thuringiensis have made it a popular and useful insecticide. First, Bacillus thuringiensis is considered extremely safe: it is harmless to humans, animals and useful insects. Second, Bacillus thuringiensis is a highly specific insecticide: most strains of Bacillus thuringiensis show toxicity to only a single order of insects

(Lepidoptera or Coleoptera or Diptera), as indicated above.

These features have also made Bacillus thuringiensis an attractive target for biotechnology and recently the new tools of this modern technique, such as gene cloning and DNA sequencing, have begun to be applied to Bacillus thuringiensis, thus creating novel Bacillus thuringiensis pesticidal proteins with either more specificity, more toxic activity or an altered range of toxicity for the host insect.

The insecticidal activity of Bacillus thuringiensis resides in the parasporal crystal (Angus, 1954). Intact crystals can be isolated from sporulated cultures of Bacillus thuringiensis by density gradient centrifugation and these isolated crystals of the Lepidopteran specific Bacillus thuringiensis variety kurstaki were shown to be composed by protein subunits of approximately 130,000 daltons (Bulla et al. 1977). In some strains such as Bacillus thuringiensis variety kurstaki HD-73 there appears to be a single protein subunit, while in other strains such as Bacillus thuringiensis HD1 there appear to be two or more very similar proteins in the crystal (Wilcox et al., 1986).

The 130.000 dalton protein is considered to be a protoxin because it is toxic to larvae only after injestion, but not after injection. The protoxin can be converted to the active toxin by digestion with proteases; and it has also been possible to isolate proteolytic fragments of the protoxin which retain full toxic activity.

Bulla et al., 1981, found that a 68,000 dalton toxin

fragment could be derived from Bacillus thuringiensis kurstaki crystals upon prolonged incubation of the solubilized protein.

Crystals of the Coleopteran specific Bacillus thuringiensis strains also appear to be composed of a single protein subunit, but of a much smaller size than the Lepidopteran protoxin.

Bernhard, 1986, isolated a 68,000 dalton protein from crystals of Bacillus thuringiensis tenebrionis, and Herrnstadt et al., 1986, observed a 64,000 dalton protein from Bacillus thuringiensis San Diego crystals. These isolated proteins are toxic upon ingestion by sensitive Coleopterans.

The crystal protein of the Lepidopteran specific Bacillus thuringiensis var. kurstaki strain, produced during the sporulation period, is also known as -endotoxin, and around 20-30% of the cell protein synthetizing activity during sporulation is devoted to the production of this toxin.

Much work has recently been directed to the isolation and characterization of genes encoding Bacillus thuringiensis toxins. The analysis of such cloned genes has already yielded important insights into toxin structure and function: it has been shown for example that genes for the crystal proteins are located on large plasmid in addition to chromosomal DNA.

Several groups have reported cloning genes for Lepidopteran specific toxins. Most of these genes have been cloned in E. coli, either utilizing antibodies to purified toxin to detect expression of the toxin, or utilizing synthetic oligonucleotide

probes based on the toxin aminoacid sequence to detect toxin genes by hybridation. The genes cloned include several genes from Bacillus thuringiensis kurstaki HD1 (Schnepf and Whiteley, 1981; Held et al., 1982; Watrud et al., 1985; Shivakumar et al., 1986; Thorne et al., 1986), and genes from Bacillus thuringiensis kurstaki HD 73 (Adang et al., 1985), from Bacillus thuringiensis sotto (Shibano et al., 1985), Bacillus thuringiensis Berliner (Klier et al., 1982; Wabiko et al., 1986), Bacillus thuringiensis aizawa (Klier et al., 1985) and Bacillus thuringiensis thuringiensis (Honigman et al., 1986). In general these genes have been shown to express toxin in E. coli and extracts of E. coli harbouring these genes are toxic to Lepidopteran larvae.

The cloned toxin genes have been used as molecular probes to determine the toxin gene number and type of many Lepidopteran active Bacillus thuringiensis strains (Kronstad et al., 1983). This analysis has shown that, while some strains (e.g. Bacillus thuringiensis kurstaki HD73) contain only a single toxin gene, many other strains contain multiple genes. Bacillus thuringiensis kurstaki HD-1 (the Dipel strain) has three distinct toxin genes (Wilcox et al., 1986).

DNA sequences and derived aminoacid sequences of the toxin proteins have been determined for several of these genes. All the genes encode proteins of between 1156 and 1178 aminoacids which are largely homologous. In some cases genes isolated from strains which had been considered distinct varieties have been found to be nearly identical. For example, the

Bacillus thuringiensis kurstaki HD-1 gene sequenced by Schnepf et al (1985) is nearly identical to the Bacillus thuringiensis sotto gene of Shibano et al. (1985). Similarly, a Bacillus thuringiensis Berliner gene (Wabiko et al., 1986) is nearly identical in sequence to another Bacillus thuringiensis kurstaki HD-1 gene. On the other hand, a third gene from Bacillus thuringiensis kurstaki HD-1 (Thorne et al. 1986) is clearly different in sequence from the two mentioned above.

Cloning and sequencing of the structural genes for the protoxin production from distinct strains of Bacillus thuringiensis kurstaki have, therefore, revealed that different related genes are responsible for the synthesis of the crystal protein toxin. These differences are evident not only among genes from different strains, but also among the multiple copies of the protoxin gene in the same strain (Andrews et al., 1987). The kurstaki HD-1 Dipel protoxin gene (Schnepf et al. 1985) and the kurstaki HD-73 protoxin gene (Adang et al., 1985) show an homology of 85% at the primary DNA sequence level. K-1 type and K-73 type crystals show distinct toxic activity against different insect species (Jaquet et al., 1987).

More particularly, with reference to the present invention, comparisons of DNA sequences encoding the crystal toxin from distinct strains of Bacillus thuringiensis have revealed the existence of both conserved and variable regions. A close look to these variable regions has shown that changes are not distributed randomly over the whole gene coding for the crystal toxin, but that differences among genes

are clustered in a hypervariable region (Geiser et al. 1986).

Only few changes or no differences have been shown at the N-terminus and the C-terminus of the crystal protein genes. In fact, from the N-terminus all genes are nearly identical for approximately the first 330 aminoacids. Similarly, from about aminoacid 600 through the C-terminus the genes are largely the same.

Optimal alignment of the DNA sequences and of the deduced polypeptide sequences of these two genes and of other Bacillus thuringiensis genes reveals that the differences are clustered in the amino terminal halves of the molecules i.e. between aminoacid residues 280 and 640 in the case of HD-1 Dipel and HD-73 genes. This region, as hereabove mentioned, is defined as hypervariable region since it shows the maximum of variability (Geiser et al., 1986; Wabiko et al., 1986; Andrews et al., 1987).

On the basis of published sequences there are at least four distinct types of Lepidopteran toxins which differ substantially in this central region. Generally, the hypervariable region is, as mentioned above, in the first half of the protoxin sequence. This hypervariable region might be the result of intramolecular recombination mechanisms between very similar, but distinct genes. Moreover, the clustering of the variable subdomains in exact regions of the crystal protein toxin, strongly suggests that the exchange of hyper variable regions between genes may have caused the large variability of biological activities of different Bacillus thuringiensis

crystal protein toxins. This variability is important in conferring toxic diversity and/or target insect range diversity among different Bacillus thuringiensis subspecies. The residual homology of the hypervariable region (in the case of HD-1/and Dipel HD-73 reduced to about 60% at the DNA level) should be sufficient to promote in vivo recombination.

A similar approach has been utilized to generate, in Escherichia coli, recombinants between human leukocyte interferon genes (EP 141484) and for Bacillus alpha amylase genes (Rey et al., 1986).

Deletion analysis of Lepidopteran toxin genes has allowed the construction of much smaller proteins with full toxicity. These truncated genes show full insecticidal activity only when the entire hypervariable region is present.

This analysis has been carried out for the Bacillus thuringiensis kurstaki HD-73 gene (Adang et al., 1985), the Bacillus thuringiensis sotto (Shibano et al., 1985), two genes from Bacillus thuringiensis kurstaki HD-1 (Schnepf and Whiteley, 1985) and a Bacillus thuringiensis Berliner gene (Wabiko et al, 1986).

Since the biological activity and the action range of the crystal toxin proteins seem to be associated to the hypervariable region sequence, and since there is a specific need to produce new specific Bacillus thuringiensis toxin, the applicant devised, according to the present invention, a novel way of generating new hybrid genes and corresponding hybrid toxin proteins, with potential different toxic specificity, by modifying the hypervariable regions of the genes

encoding the crystal protein toxins.

Modification of the hypervariable regions of the crystal protein encoding genes could be done by site-specific mutagenesis, a technique consisting basically in introducing specific nucleotide mutations, either substitutions or deletions, in the crystal toxin hypervariable gene region, in order to obtain aminoacid substitutions in the polypeptide chain encoded by such mutagenized DNA sequence.

This technique, nevertheless, is in general very useful and efficient only if the molecular mechanism of action of the protein to be modified is known. The tridimensional structure of the protein should be known as well to be able to forecast what could be the consequences of the specific aminoacid substitutions or deletions in the protein to be modified. With reference to the Bacillus thuringiensis crystal toxin protein there are no data available as to its tridimensional structure and this is due at least to two main reasons:

1) It is quite difficult to obtain crystals of the toxin protein which can be used in a crystallographic analysis.

2) Last but not least, the size of the polypeptide chain (135,000 dalton) would make the crystallographic analysis very laborious.

As a consequence, the site-specific mutagenesis does not seem to be very useful in providing an easy and efficient means to obtain new Bacillus thuringiensis crystal toxins.

Another alternative approach could be a mutagenesis conducted at random on a Bacillus thuringiensis

crystal toxin cloned gene, but this method, lacking in specificity, does not seem to be useful to obtain new toxins characterized by insecticidal activities, since mutations introduced in the toxin gene according to mutagenesis conducted at random only affect a limited number of aminoacid residues.

There is therefore a specific need to provide an easy and efficient method capable of producing new Bacillus thuringiensis crystal toxin by modifying the hypervariable region of the crystal toxin gene.

Accordingly, the present invention, by means of an in vivo-recombination process, provides a potentially unlimited number of new hybrid genes coding for new corresponding hybrid crystal protein toxins having either different insecticidal activities and/or an alterated target insect range.

The cited prior art documents are identified hereinafter:

Adang M.J., Staver M.J. Rochleau T.A., Leighton J., Barker R.F., Thompson D.V., (1985). Characterized full length and truncated clones of the crystal protein of Bacillus thuringiensis subsp. kurstaki HD73 and their toxicity to Manduca sexta. Gene 36,289-300.

Andrews R.S., Faust R.M., Wabiko H., Raymond K.C., Bulla L.A., (1987). The biotechnology of Bacillus thuringiensis. CRC Critical Reviews in Biotechnology, 6, 163-232

Angus T.A., (1954). A bacterial toxin paralysing silkworm larvae. Nature 173, 545-546

Angus T.A., (1956). Extraction, purification and properties of Bacillus sotto toxin. Can. J.Microbiol.

2, 416-426

Bernhard K., (1986). Studies on the delta endotoxin of Bacillus thuringiensis Var. tenebrionis. FEMS Microbiol.Lett. 33, 261-265

Birnboim H.C., Doly J., (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Res. 7, 1513-1523

Bulla L.A., Kramer K.J., Cox D.J., Jones B.L., Davidson L.I., Lookhart G.L., (1981). Purification and characterization of the entomocidal protoxin of Bacillus thuringiensis. J. Biol. Chem. 256, 3000-3004

Bulla L.A., Kramer K.J., Davidson L.I., (1977). Characterization of the entomocidal parasporal crystal of Bacillus thuringiensis. J. Bacteriol. 130,375-383

Dulmage H.T., (1981). Insecticidal activity of isolates of Bacillus thuringiensis and their potential for pest control, pp. 193-222. In Burges H.D. (ed.) Microbial control of pests and plant diseases 1970-1980.
Academic Press New York.

Geiser M., Schweitzer S., Grimm C., (1986). The hypervariable region in the genes coding for entomopathogenic crystal protein of Bacillus thuringiensis: nucleotide sequence of the kurhdl gene subsp. kurstaki HD1. Gene 48, 109-118.

Goldberg L.J., Margalit J., (1977). A bacterial spore demonstrating rapid larvicidal activity against Anopheles sergentii, Uranatenia unguiculata, Culex univittatus, Aedes aegypti and Culex pipiens. Mosquito News 37, 353-358.

Hanahan D., (1985). Techniques for

transformation of E. coli. In DNA cloning (D.M. Glover, ed.), volume 1, pp. 109-132. IRL Press Oxford-Washington D.C.

Held G.A., Bulla L.A., Ferrari E., Hoch J., Aronson A.I., Minnich S.A., (1982). Cloning and localization of the lepidopteran protoxin of Bacillus thuringiensis subsp. kurstaki. Proc. Nat. Ac. Sci. USA 79, 6065-6069.

Herrnstadt C., Soares G.G., Wilcox E.R., Edwards D.L. (1986). A new strain of Bacillus thuringiensis with activity against Coleopteran insects. Biotechnology 4, 305-308.

Honigman A., Nedjar-Pazerini G., Yawetz A., Oron U., Schuster S., Broza M., Sneh B., (1986). Cloning and expression of the Lepidopteran toxin produced by Bacillus thuringiensis var. thuringiensis in E. coli. Gene 42, 69-77.

Jaquet F., Hutter R., Luthy P., (1987) Specificity of Bacillus thuringiensis δ-endotoxin. Applied and Environmental Microbiology 53, 500-504.

Klier A., Fargette F., Ribier J., Rapoport G., (1982). Cloning and expression of the crystal protein genes from Bacillus thuringiensis strain berliner 1715. EMBO J., 1, 791-799.

Klier A., Lereclus D., Ribier J., Bourgouin C., Menou G. Lecadet M., Rapoport G., (1985). Cloning and expression in E. coli of the crystal protein gene from Bacillus thuringiensis strain aizawa 7-29 and comparison of the structural organization of genes from different serotypes, pp. 217-224. In Hoch. J.A., Setlow P. (eds.) Molecular biology of microbial differentiation. American Society for Microbiology

Washington.

Krieg A., Huger A.M., Langenbruch G.A., Schnetter W., (1983). Bacillus thuringiensis var. tenebrionis, a new pathotype effective against larvae of Coleoptera. Z. Angew. Entomol. 96, 500-508

Krieg A., Huger A.M., Langenbruch G.A., Schnetter W., (1984). New results on Bacillus thuringiensis var. tenebrionis with special regard to its effect on Colorado potato beetle. Anz. Schaedlingskd. Pflanzenschutz Umweltschutz 57, 145-150.

Krieg A., Huger A.M., Schnetter W., (1987). Bacillus thuringiensis var. san diego Stamm M-7 ist identisch mit dem zuvor in Deutschland isolierten Käferwirksamen B. thuringiensis subsp. tenebrionis Stamm BI 256-82.

Kronstad J.W., Schnepf H.E., Whiteley H.R., (1983). Diversity of location for Bacillus thuringiensis crystal protein genes. J. Bacteriol. 154, 419-428.

Kronstad J.W., Whiteley H.R. (1984). Inverted repeat sequences flank a Bacillus thuringiensis crystal protein gene. Journal of Bacteriology 160, 95-102.

Rey M.W., Requadt C., Mainzer S.E., Lamsa M.H., Ferrari E., Lad P.J., Gray G.L. (1986). Homologous α-amylases of Bacillus and generation of their hybrids in vivo. In Bacillus Molecular Genetics and Biotechnology Applications (A.T. Ganesan and J.A. Hoch, eds.), pp. 229-239. Academic Press, Orlando - London.

Sanger F., Nicklen S. Coulson A.R., (1977). DNA

Sequencing with chain terminating inhibitors. Proc. Natl. Acad. USA 74, 5463-5467.

Schnepf H.E., Wong H.C., Whiteley H.R. (1985). The aminoacid sequence of a crystal protein from Bacillus thuringiensis deduced from the DNA base sequence. J. Biol. Chem. 260, 6264-6272.

Schnepf H.E., Whiteley H.R. (1981). Cloning and expression of the Bacillus thuringiensis crystal protein gene in E. coli Proc. Nat. Acad. Sci. USA 78, 2893-2897.

Schnepf H.E., Whiteley H.R, (1985). Delineation of toxin encoding segment of a Bacillus thuringiensis crystal protein gene. J. Biol. Chem. 260, 6273-6280.

Shibano Y., Yamagata A., Nakamura N. Iizuka T. Sugisaki H., Takanami M., (1985). Nucleotide sequence coding for the insecticidal fragment of the Bacillus thuringiensis crystal protein. Gene 34, 243-251.

Shivakumar A.G., Gundling G.J. Benson T.A., Casuto D., Miller M.F. Spear B.B., (1986). Vegetative expression of the delta endotoxin genes of Bacillus thuringiensis subsp. kurstaki in B. substilis. J. Bacteriol. 166, 194-204.

Thorne L., Garduno F., Thompson T., Decker D., Zounes M., Wild M., Walfield A.M., Pollock T.J., (1986). Structural similarity between the Lepidoptera and Diptera specific insecticidal endotoxin genes of Bacillus thuringiensis subsp. kurstaki and israelensis. J. Bacteriol. 166, 801-811.

Wabiko H., Raymond K.C., Bulla L.A., (1986). Bacillus thuringiensis entomocidal protoxin gene sequence and gene product analysis. DNA 5, 305-314.

Wartrud L.S., Perlak F.J., Tran M.T., Kusano K.,

Mayer E.J., Miller-Wideman M.A., Obukowicz M.G., Nelson D.R., Kreitinger J.P., Kaufman R.J., (1985). Cloning of the Bacillus thuringiensis subsp. kurstaki delta endotoxin gene into Pseudomonas fluorescens: Molecular biology and ecology of an engineered microbial pesticide, pp. 40-46. In Halvorson H.O., Pramer D., Rogul M. (eds.) Engineered organisms in the environment. American Society for Microbiology Washington.

Weber H., Weissmann C., (1983). Formation of genes coding for hybrid proteins by recombination between related, cloned genes in E. coli. Nucleic Acid Research 11, 5661-5669.

Wells J.A., Ferrari E., Henner D.J., Estell D.A., Chen E.Y., (1983). Cloning sequencing and secretion of B. amyloliquefaciens subtilisin in B. subtilis. Nucleic Acid Research 11, 7911-7925.

Whiteley H.R., Schnepf H.E. (1986). The molecular biology of parasporal crystal body formation in Bacillus thuringiensis. Annual Review of Microbiology 40, 549-576.

Wilcox D.R., Shivakumar A.G., Melin B.E., Miller M.F., Benson T.A., Schopp C.W. Casuto D., Gundling G.J., Bolling T.J., Spear B.B., Fox J.L. (1986). Genetic engineering of bioinsecticides, pp. 395-413. In Inouye M., Sarma R. (eds.) Protein engineering. Academic Press New York.

The present invention concerns new hybrid Bacillus thuringiensis genes, obtained by in vivo recombination, encoding new corresponding Bacillus thuringiensis hybrid crystal protein toxins having either different insecticidal acivities and/or an

altered insect host range.
hybrid
The new/ Bacillus thuringiensis genes object of the present invention are obtained, as above mentioned, by means of in vivo recombination of the hypervariable region present in the Bacillus thuringiensis genes coding for the Bacillus thuringiensis crystal protein toxins.

The present invention also comprises new polypeptides, e.g. new hybrid crystal protein toxins obtained by in vivo recombination of the hypervariable region of two genes coding for the Bacillus thuringiensis crystal protein toxins.

The two genes to be recombined in vivo can be:

a) derived from natural Bacillus thuringiensis strains

b) they can be the products of a previous in vivo recombination event.

Another embodiment of the present invention refers to a novel process of production of new hybrid pesticidal toxins, in particular Bacillus thuringiensis crystal protein toxin, by in vivo recombination of the hypervariable regions of genes coding for a pesticidal toxin, said genes having enough residual homology to be able to recombine in vivo.

In a preferred embodiment of this invention the two hypervariable regions to be recombined in vivo come from two different genes of Bacillus thuringiensis kurstaki and more particularly one gene is the crystal toxin encoding gene from Bacillus thuringiensis kurstaki HD-1 Dipel (Gene HD-1) and the other gene is the crystal toxin encoding gene from

Bacillus thuringiensis kurstaki HD-73 (Gene HD-73).

According to the present invention the in vivo recombination process applies to crystal protein encoding genes isolated from the following strains:

Bacillus thuringiensis alesti
aizawai
canadensis
dakota
darmstadiensis
dendrolimus
entomocidus
finitimus
fowleri
galleriae
indiana
israelensis
Keniae
kurstaki
kyushuensis
morrisoni
ostriniae
pakistani
San Diego
sotto
tenebrionis
thompsoni
thuringiensis
Bacillus thuringiensis tolworthi
toumanoffi
wuhanensi

The present invention also refers to plasmid vectors which contain two genes encoding a pesticidal protein

toxin or a part thereof, said genes having enough residual homology to be able to recombine in vivo.

In an embodiment of the present invention these genes are the genes encoding the Bacillus thuringiensis crystal protein toxin.

In a preferred embodiment of the present invention these genes are the genes encoding the Bacillus thuringiensis variety kurstaki crystal protein toxin and in a more preferred embodiment of the present invention these genes are the genes encoding the Bacillus thuringiensis variety kurstaki HD-1 Dipel crystal protein toxin (gene HD-1) and the Bacillus thuringiensis variety kurstaki HD-73 crystal protein toxin (gene HD-73).

These plasmid vectors containing the Bacillus thuringiensis DNA sequences to be recombined in vivo (for the reasons explained below they might be called "father plasmid vectors") represent a source of a potentially unlimited number of plasmid vectors ("son plasmid vectors") wherein the Bacillus thuringiensis DNA sequences have recombined in vivo and now these new hybrid DNA sequences encode new Bacillus thuringiensis hybrid crystal toxins.

The present invention also refers to plasmid vectors ("son plasmid vectors" as above mentioned) containing new Bacillus thuringiensis hybrid DNA sequences, resulting from in vivo recombination of two Bacillus thuringiensis genes encoding the crystal protein toxin wherein these new hybrid DNA sequences encode new Bacillus thuringiensis hybrid crystal toxins having either different insecticidal activities and/or an altered insect host range.

Objects of the present invention also are expression vectors containing the new hybrid Bacillus thuringiensis DNA sequences obtained according to this inventions and regulatory functions (like promoters, attenuators, ribosome binding sites, specific SHINE-DALGARNO sequences, stop codons, enhancers) which allow a very high expression of said new hybrid Bacillus thuringiensis DNA sequences and therefore a high production of their corresponding hybrid polypeptides.

Expression controlling sequences useful in expressing the new hybrid B. thuringiensis DNA sequences of this invention include, but are not limited to, the lac system, trp system, the major operator and promoter regions of phage λ, the control regions of fd-coat protein, the β-lac system, the TAC system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses or combination thereof.

Another embodiment of the present invention refers to host cells transformed by plasmid vectors as here above described.

Hosts useful for preparation of the hybrid DNA sequences of the present invention by in vivo recombination include various strains of E. coli, Pseudomonas, B. subtilis, B. thuringiensis, Agrobacterium, yeasts.

For the transformation, for obtaining the hybrid Bacillus thuringiensis DNA sequences of the present invention, host cells can be recombination-proficient, $recA^+$.

Moreover, the in vivo recombination process of

Bacillus thuringiensis DNA sequences encoding crystal toxin protein occurs in host cells rec A^-, rec A^- strains can also be used.

the use of $recA^-$ strains for in vivo recombination provides a further differentiation from the one described by Weissmann and Weber in EP-141484.

In a preferred embodiment of the present invention Escherichia coli host cells have been used.

Two father plasmid vectors called pT173 and pGEM173 were transformed in Escherichia coli HB 101, so giving rise to strains I-879 and I-878, filed at Paris Pasteur Institute on June 28, 1989. The skilled persons in the art can understand that several other host cells may be used.

Host cells useful for the expression of the hybrid Bacillus thuringiensis DNA sequences of the present invention are:

E. coli

Bacillus different species

Bacillus thuringiensis

Agrobacterium

Yeasts

Baculoviruses

Rhizobium

The present invention also comprises the use of the new hybrid Bacillus thuringiensis DNA sequences, object of this invention, for the preparation of new plasmid vectors containing these new hybrid Bacillus thuringiensis DNA sequences, wherein said plasmid vectors are used to transform plant cells.

New hybrid Bacillus thuringiensis DNA sequences of the present invention coding for new hybrid Bacillus

thuringiensis crystal protein toxins can in fact be engineered into plant cells to yield insect resistant plants.

The methodology for engineering plant cells is well established (see Nester E.W., Gordon M.P., Amasino R.M. and Yanofsky M.F., Ann. Rev. Plan. Physiol. 35:387-399, 1984;and EP 142 924).

Plant cells transformed by these plasmid vectors are also within the scope of the present invention.

The present invention comprises as well transgenic plants containing new hybrid Bacillus thuringiensis DNA sequences as produced according to this invention.

In addition to be used to transform plant cells, the new hybrid Bacillus thuringiensis DNA sequences of the present invention can be introduced into microorganisms capable of occupying, surviving and proliferating in the phytosphere of plants according to the procedures disclosed in EP-0200344.

The present invention comprises as well muteins of the hybrid Bacillus thuringiensis protein sequences of this invention, wherein these muteins have been obtained by standardized genetic engineering techniques (like site-specific mutagenesis, random mutagenesis, glycosilation) and their activity is reconducible to the insecticidal activity of their parent hybrid Bacillus thuringiensis crystal toxin proteins.

The present invention also comprises new pesticidal compositions containing new hybrid Bacillus thuringiensis polypeptides obtained according to this invention, in combination with suitable eccipients,

adjuvants and aggregants etc. These compositions are prepared by intimately and uniformly mixing the new hybrid Bacillus thuringiensis polypeptides of the present invention with suitable finely divided diluents, fillers, eccipients, disintegrating agents and the like.

As to the process of obtaining in vivo recombination of DNA homologous sequences we referred to a method developed by Weber and Weissman (1983) and EP-141484.

The methods of their invention are characterized by the steps of a) preparing a DNA fragment or concatemer thereof, said fragment comprising in sequence one of the parental DNA sequences from which the hybrid DNA is to be derived, an intact replicon such that the DNA fragment may be replicated in a host cell, and the other parental DNA sequence from which the hybrid DNA sequence is to be derived, the two parental DNA sequences having sufficient homology to promote their recombination in vivo; and b) selecting host cells that have been transformed with the desired hybrid DNA sequence and isolating said hybrid DNA sequence from them. Selection of the desired host cells may be facilitated by having each parental DNA sequence associated with a different antibiotic resistance marker, and growing the transformed host cells on agar plates containing both antibiotics.

The hybrid DNA sequences produced by these methods clearly enable the production of novel hybrid polypeptide having a variety of uses and biological activities.

According to EP 141484, these methods were originally

used to produce new hybrid interferons genes by in vivo recombination of two interferon genes α-1 and α-2 having partial sequence homology (80%).

Thus DNA structures consisting of plasmid vector sequences flanked by the α-2 interferon gene on the one side and a portion of the α-1 interferon gene on the other were transfected into E. coli host cells. Appropriate resistance markers allowed the isolation of colonies containing circular plasmides which arose by in vivo recombination between the partly homologous interferon gene sequences. In the plasmid vectors different recombinant genes were identified, all of them encoding for new hybrid interferon not easily accessible by traditional recombinant DNA techniques. This method, according to EP 141484, should be generally applicable to the formation of recombinants between not too distantly related genes. But, while according to the method described by Weissmann and Weber in EP-141484 host cells should be recombination-proficient $recA^+$, we have recently found that in vivo recombination of the hypervariable regions of Bacillus thuringiensis genes encoding the Bacillus thuringiensis crystal protein toxin occurs in host cells as well that are $recA^-$. Furthermore the experimental process of the applicant thanks to the use of father plasmid vector, is shorter, safer and less difficult than that described by Weber and Weismann in EP-141484.

With a technique similar to that disclosed in Ep-141484 Rey et al., 1986, obtained recombinant α-amylases by in vivo recombination between the genes coding the B. licheniformis α-amylase and the

homologous gene coding the B. stearothermophilus α-amylase.

Another way of producing novel toxins or altering the insect host range of Bacillus thuringiensis toxins is provided by a method, described in EP 228838 herein enclosed as a reference, which comprises recombining in vitro the variable regions of two or more δ-endotoxin genes.

Specifically exemplified in EP 228838 is the recombining of variable regions to two Bacillus thuringiensis kurstaki sequences, in particular HD-1 and HD-73, to produce chimeric Bacillus thuringiensis δ-toxins with altered ranges as compared to the toxins produced by their parent DNA.

Variable regions, as used in that patent application, refers to non-homologous regions of two or more B.t. δ-DNA sequences which upon in vitro recombination yields a DNA sequence encoding a new δ-endotoxin with an altered insect host range. According to the method described in EP 228838, two Bacillus thuringiensis gene showing partial homology are recombined in vitro by first cutting and then religating with restriction enzymes specific Bacillus thuringiensis DNA sequences in order to obtain recombination of the non-homologous regions.

However, even if this method is quite specific and allows production of new Bacillus thuringiensis toxins, it suffers as well of a considerable drawback since, according to an in vitro recombination process, only a limited amount of hybrid proteins can be produced. The method of EP 228838 requires in fact the identification of specific restriction sites in

both the genes to be recombined, while the method of the present invention, wherein recombination is carried out in vivo, allows the production of a potentially unlimited number of hybrid Bacillus thuringiensis toxins.

The disclosure of the present invention is intended to be read in conjunction with the references cited which are set forth in the appended bibliography.

The following examples which illustrate procedures, including the best mode to practice the invention, should not be considered limiting. The examples are for illustration purpose and intended to describe this invention so that it may be clearly understood.

The present invention provides new hybrid Bacillus thuringiensis DNA sequences obtained by in vivo recombination of two or more different genes encoding Bacillus thuringiensis crystal protein toxins.

According to the present invention, therefore, we have subcloned in plasmid vectors two truncated genes of Bacillus thuringiensis variety kurstaki.

The source of the HD-1 Dipel gene portion was the plasmid pESAC, a derivative of pES1 (ATCC 31995) described by Schnepf and Whiteley (1981). The HD-73 gene portion derived from pJWK20 plasmid (ATCC 31997) described by Kronstad and Whiteley, 1984.

pBS19 is an E. coli - B. subtilis shuttle vector derived from pBS42 (Wells et al., 1983; Greg Gray, unpubl.).

The gene obtained from plasmid pES1 of Bacillus thuringiensis variety kurstaki HD-1 (gene HD-1 Dipel) consisted in the promoter, the 5' coding sequence and the whole hypervariable coding region.

The second gene (gene HD-73) obtained from plasmid pJWK20 (Adang et al., 1985) of Bacillus thuringiensis variety kurstaki HD-73 was truncated in the 5' coding region and consisted, therefore, of the hypervariable region and the 3' terminal coding sequence.

These two genes, gene HD-1 Dipel and gene HD-73, are among those isolated from Bacillus thuringiensis variety kurstaki showing the greatest differences at the level of the nucleotide sequence of the hypervariable region. Crystal protein toxins produced by these two different bacterial serotypes Bacillus thuringiensis kurstaki HD-1 and Bacillus thuringiensis kurstaki HD-73 show different insecticidal activity for different species of Lepidopterans.

According to the present invention the two truncated genes here above described have been cloned into plasmid pBS19 which contains a gene capable of conferring the chloramphenicol resistance phenotype. Between the two cloned Bacillus thuringiensis variety kurstaki genes, namely gene HD-1 Dipel and gene HD-73, a marker gene coding for tetracycline resistance was inserted. The result of this construction is plasmid vector pT173 which is shown in Figure 1.

Another plasmid, corresponding to plasmid pT173 and called pGEM 173 (10.8 Kb) is shown in Figure 2.

This plasmid has been obtained inserting a truncated fragment of both the Bacillus thuringiensis kurstaki HD-1 gene and the Bacillus thuringiensis kurstaki HD-73 gene in the plasmid vector pGEM4Z (Promega, Madison, WI, USA Plasmid pT173, once introduced in Escherichia coli cells synthetizes a polypeptide of 65

Kd which is recognized by specific antibodies for the Bacillus thuringiensis crystal toxin protein. This polypeptide has been identified as the product of Bacillus thuringiensis variety kurstaki HD-1 which, as here above described, is truncated downstream of the hypervariable region. The partial homology between the two hypervariable regions of gene HD-1 Diepel and gene HD-73 (at nucleotide sequence level this homology is 62,2%) should be sufficient to be able to promote an in vivo recombination process of the two truncated genes.

It is clear that since this is an in vivo recombination process, plasmid vectors like plasmid pT173 can produce an unlimited number of different recombinant hybrid Bacillus thuringiensis DNA sequences encoding for their corresponding hybrid Bacillus thuringiensis polypeptides having either different insecticidal activity and/or an altered target insect range.

Plasmid pT173, therefore, has been transformed in suitable Escherichia coli host cell.

In one embodiment of the present invention, these Escherichia coli host cells are recombination proficient rec A^+, but according to some more recent results, in vivo recombination of Bacillus thuringiensis DNA sequences occurs also in cells which are rec A^-.

The recombination deficient rec A^- cells (instead of recombination proficient rec A^+) have the advantage that the hybrid DNA is not rearranged. In this way accuracy of recombination is obtained at the DNA level, avoiding casual rearrangment and then the

occurence of unexpected and unwanted sequences.

After many generations plasmid DNA is extracted and digested with the restriction enzyme Nru I.

Since recombinant plasmid carrying new Bacillus thuringiensis hybrid DNA sequences obtained upon in vivo recombination have lost the DNA restriction site recognized by Nru I (as it will be explained in a more detailed way further on), treatment with the endonuclease Nru I allows selection of plasmids which have recombined in vivo.

These recombinant plasmids, being the only ones still circular after the Nru I treatment, will be able successively to transform E. coli cells producing their colonies which will be resistant to chloramphenicol.

The same procedure applies to plasmid pGEM 173, with the only difference that E. coli transformants are selected for resistance to ampicillin instead of chloramphenicol.

According to the method hereabove described, we succeeded in isolating many different recombinants whose hybrid DNA sequences are reported in Figure 6 a - e.

MATERIALS AND METHODS

BACTERIAL STRAINS

The following bacterial strains and their rec A derivative were used for transformation:

Bacterial strains

Escherichia coli strains were: HB101 (F- hsdS20 recA13 ara-14 proA2 leuB6 lacY1 galK2 rpsL20 xyl-5 mtl -l sup E44)

JM103: (lac proAE) Δ (lac pro), thi, strA, supE,

endA, sbeB, hsdR$^-$, F'traD36, proAB, lac19, ZΔM15

294 endA thi pro hsdR hsdM hsm

294recA endA thi pro hsdR hsdM hsm recA

Escherichia coli host cells were made competent and transformed according to Hanahan 1985.

CULTURE MEDIA

LB medium (per liter: Difco Bacto-tryptone 10g; Difco Bacto-yeast extract 5g; NaCl 5g)

For growth of strains with plasmid pT173, tetracycline 12.5 µg/ml or chloramphenicol 10 µg/ml were added to LB medium.

For growth of strains with plasmid pGEM-173 ampicillin was used at 100 µg/ml.

PLASMID DNA EXTRACTION

Plasmid DNA preparation has been done by the alkaline lysis method adapted to larger samples and followed, for sequencing, by a PEG precipitation (Birnboim and Doly, 1979).

RESTRICTION ENZYMES, DIGESTION, ELECTROPHORESIS

Restriction enzymes were from Boehringer Mannheim GmbH II (FRG) and from Bethesda Research Laboratories (Maryland, USA).

Restriction enzymes digestion, ligation and other treatments during plasmid construction have been done following the suggested protocols of the supplier.

Restriction patterns generated from the digestion of plasmid DNA were resolved on 0,7% agarose gels (agarose from Bethesda Research Laboratories). DNA samples were electrophoresed in TBE buffer (0,3M Tris-borate pH 8.3, 2 mM EDTA) and stained with ethidium bromide. When necessary electrophoresis was performed on acrylamide gels (6%) in TBE buffer.

DNA SEQUENCE ANALYSIS

Fragments of DNA, originated from recombinant plasmids derived from pT173 and pGEM-173, were obtained by HindIII digestion. A band corresponding to DNA of approximately 2.9 Kb was separated by agarose gel electrophoresis, recovered by electroelution and subsequently digested with EcoRV. A band of DNA of approximately 700 bp was purified by acrylamide gel electrophoresis (6%) electroeluted and ligated to the plasmid pGEM-4Z (Promega, Madison, W1, USA) digested with SmaI and treated with phosphatase. Sequencing was performed by the chain termination method of Sanger et al. (1977) adapted to plasmid DNA (Chen and Seeburg, 1985).

Sequenase (United States Biochemical) is known, in the dideoxi-chain elongation reaction.

IMMUNOBLOTTING

The method of Towbin et al. (1974) was used to detect the crystal protein immunologically.

Proteins resolved by Sodium dodecyl sulphatepolyacrylamide gel electrophoresis were transferred electrophoretically to nitrocellulose sheets washed with 50 mM Tris hydrochloride-200 mM NaC1 containing 0.1% Nonidet P-40 and then incubated with the antiserum.

After a wash with the same buffer, the nitrocellulose sheets were incubated with peroxidase-conjugated sheep anti-rabbit immunologlobulin G antiserum (United States Biochemical Co., Cleveland, Ohio).

The immuno-complexes were then visualized in the presence of hydrogen peroxide and 4-chloro-1-naphtol as substrates (GIBCO Laboratories, Grand Island

N.Y., Bethesda Research Laboratories, Inc. Gaithersburg, Md.)

PLASMID CONSTRUCTION

pT is a pBS19 derivative: a 1424 bp Eco RI - Ava I (with even ends in Ava I) fragment of pBR322 bearing tetracycline resistance was inserted in pBS19 digested with Eco RI and Sac I (the latter digestions followed by treatments able to obtain even ends).

pT73 is a pT derivative obtained by inserting in the Eco RI site of pT an Eco RI fragment of approximately 5400 bp obtained from pJWK20 (Fig. 1). The fragment comprises the last two thirds of the HD73 toxin gene starting from residue 1383 of the sequenced region (Adang et al., 1985). pT1 was obtained as follows: pT was digested to completion with Sma I, partially with Bam HI and ligated to a fragment of approximately 1900 bp derived from pESAC and corresponding to the first portion of HD-1 Dipel toxin gene from nucleotide residue 291 to residue 2215 of the sequenced region (Schnepf et al., 1985).

The fragment was obtained by digestion of pESAC with Hind III followed by treatment with Klenow to make it blunted and then by restriction with Bam HI.

pT173, the plasmid used for the in vivo construction of recombinant genes, derived from the insertion of the 2500bp Bam HI-Bam HI fragment of pT1, comprising the first part of the HD-1 Dipel gene and the last two thirds of the resistance tetracycline gene, in pT73 completely cleaved with Bam HI and dephosphorilated with pancreatic phosphatase to avoid re-insertion of the original Bam HI fragment.

Tet^R and Cm^R transformants obtained in E. coli 294

racA strain were examined by plasmid extraction and restriction analysis.

Plasmids pT1, pT73 and pT173 were not able to direct the synthesis of a complete endotoxine polypeptide but only of a 65-68 kdal fragment which immunoreacted with antibodies raised against the pure toxic crystal in pT1 and pT173.

The tetracycline resistant gene was inserted in inverted orientation to ensure that the HD73 truncated sequence could not be expressed from external expression control regions in pT73 and pT173.

GENERATION OF HYBRID GENES

The sequences of HD-1 Dipel and HD73 genes in pT173 share a region of homology, i.e. the last 696 bp of HD-1 Dipel sequence and the first 707 bp of the HD-73 sequence. Between these partial direct repeats there is the tetracycline resistance determinant characterized by the unique Nru I site.

Figure 3 shows the protocol followed to generate the recombinant plasmids with the hybrid toxin genes.

The pT173 plasmid was introduced in a recombination proficient background by transformation of E. coli 294 competent cells.

A single colony Tet^R and Cm^R has been inoculated in LB (supplemented with chloramphenicol) and grown for about 40 generations. Plasmid DNA extracted from the cells was digested with Nru I. In this way the molecules not subjected recombination and having the intact tetracycline gene were linearized. Only circular molecules could replicate and transform the 294 recA competent cells to Cm^R.

This allows selection of plasmids which have recombined in vivo: they have lost the restriction site recognized by the endonuclease Nru I and according to that they remain circular.

The Cm^R transformants were screened for sensitivity to tetracycline with the aim of obtaining recombinants between the partially homologous regions of the truncated toxin genes (boxed in Figure 3). In this way we expected to reconstitute an entire hybrid gene with the first third of the aminoterminal region of the HD-1 Dipel gene and two thirds from the carboxyterminal region of the HD-73 gene. The hypervariable region was expected to be a different hybrid region for each clone able to express a polypeptide immunoreacting with specific antibodies.

We isolated 13 Cm^R Tet^S clones, examined them for the production of a polypeptide immunoreacting with polyclonal antibodies raised against HD-73 toxic crystals and for the presence of recombinant plasmids (pTHy).

ANALYSIS OF THE RECOMBINANT OBTAINED

Escherichia coli cells transformed with the recombinant plasmids have been analyzed by immunoblotting to identify all the clones capable of synthesizing a polypeptide chain of 135 kd having the immunological properties of the Bacillus thuringiensis crystal toxin protein. The method of Towbin et al (1974) was used to detect the crystal protein immunologically.

According to this method 10 positive clones were identified.

In order to characterize these positive clones

identified, recombinant plasmids have been purified and their DNA was digested with restriction enzymes in order to obtain their restriction maps. Plasmid purification and restriction enzyme digestion have been done according to the methods indicated previously in description of the present patent application.

All the plasmids analyzed were identified as recombinant plasmids obtained by in vivo recombination of the hypervariable regions of the two Bacillus thuringiensis kurstaki genes contained either in plasmid pT173 or pGEM173.

These two "father plasmids" are in fact sources of a potentially unlimited number of new hybrid Bacillus thuringiensis DNA sequences obtained by in vivo recombination.

All these recombinant plasmids were further characterized by determining the nucleotide sequence of DNA fragments corresponding to the hypervariable regions wherein, according to the present invention, recombination has occurred.

DNA sequence analysis has been done according to the method indicated in the description of this patent application.

In figure 4 the HD-1 and the HD-73 Bacillus thuringiensis DNA sequences are aligned to maximize the matches (represented by a vertical line). The regions of cross-over are indicated by a box.

While most of the recombinant hybrid DNA sequences obtained and identified are different from each other, some of them were completely identical and we can not exclude that they might be "brothers"

resulting from a unique recombination process.

As indicated in Figure 4, the in vivo recombination process has occurred through all the hypervariable region. So far, of all the recombinant hybrid Bacillus thuringiensis DNA sequences analyzed, excluding those which recombined in the same region, 10 hybrids used a different region of cross-over. The DNA sequences of these 10 new recombinant hybrid Bacillus thuringiensis genes are reported in Figure 6 a - e.

These recombinant hybrid sequences have been called respectively: HY45, HY3, HY6, HY53, HY21, HY32, HY2, HY 127, HY 126, HY 5.

The aminoacid sequence of the proteins coded by these recombinant hybrid Bacillus thuringiensis genes can be easily deduced from their nucleotide sequence.

A comparison of the deduced aminoacid sequences is reported in Figure 5 which represents the optimal alignment of these polypeptide products deduced from the DNA sequence of their parental recombinant hybrid genes. According to the specific site where the recombination process has occurred, the hybrid protein corresponds in the hyper variable region either to the protein encoded by the gene HD-1 Dipel or to the protein encoded by the gene HD 73.

Two cases have been identified, HY6, HY64 and HY 127, HY 21, where, while the hybrid recombinant genes have a different nucleotide sequence, the polypeptide chains are identical. At the aminoacid level, therefore, the real number of new recombinants encoding hybrid Bacillus thuringiensis crystal protein toxins is eight. These hybrid genes encode

hybrid proteins which are different from their parental natural Bacillus thuringiensis crystal toxin proteins and from any other known Bacillus thuringiensis crystal toxin protein.

Accordingly, these new hybrid proteins might have either different insecticidal activities and/or an altered insect host range as compared to the natural crystal toxin proteins produced by Bacillus thuringiensis variety kurstaki HD-1 Dipel or Bacillus thuringiensis variety kurstaki HD-73.

The experimental procedure described in the present invention in order to generate hybrid recombinants between different Bacillus thuringiensis genes can be applied to other couples of Bacillus thuringiensis genes or to different recombinant products, generating new polypeptides possibly having spectra of toxic activity different from that of the parental products. Among the 8 new different hybrid Bacillus thuringiensis crystal protein toxins isolated, preliminary results indicate that some of them have a specific toxic activity against Ostrinia nubilalis larvae (European corn borer).

It is clear to those skilled in the art that direct sequence analysis of other recombinants combined with assays of toxicity against different Lepidopteran targets could lead to the identification of new toxins with either a different insecticidal activity or an altered insect host range. Those skilled in the art will appreciate, therefore, that the invention described herein and the methods of practising it specifically described are susceptible of variations and modifications other than as specifically

described.

It is to be understood that the invention includes all these variations and modifications which are intended to be fully within the scope of the following claims.

The numerals set forth below represent figure numbers for the appended drawings.

Fig. 1 - Shows the construction of pT173 plasmid.

PT is a derivative of pBS19 able to express chloramphenicol resistance (Cm) and tetracycline resistance (Tet, heavy black arrow) in E. coli and B. subtilis.

The open box is the HD-73 toxin coding sequence starting from residue 1383. The direction of transcription is indicated. The heavy line represents the HD-73 sequences downstream the toxin gene.

Dashed box represents the HD-1 Dipel sequence starting from residue 291 to residue 2215. The direction of transcription is indicated. The interrupted arrows indicate the region of partial homology.

Fig. 2 - Shows the restriction map of plasmid pGEM173 (10.8 kb)

This plasmid is obtained by inserting a truncated fragment of the HD-73 gene, more particularly an EcoRI-ecoRI fragment derived from plasmid pJWK20 (as for the construction of plasmid pT173) in the plasmid vector pGEM 4Z Promega.

The truncated HD-1 gene inserted in plasmid pGEM-173 is a BamHI-BamHI obtained from plasmid pT173. This BamHI-BamHI fragment only includes part of the Tct^R

gene, which contains a NrvI restriction site.

Fig. 3 - Is a schematic representation of the process of in vivo recombination of the present invention.

Plasmid PT 173 (13 kb) carrying the Bacillus thuringiensis HD-1 and HD-73 truncated sequences, separated by the marker gene for the resistance to tetracycline, has been introduced by transformation in E. coli ($RecA^+$) cells wherein in vivo recombination of the two Bacillus thuringiensis DNA sequences occurs.

PTHY represents isolated recombinant vectors carrying genes coding for a new hybrid Bacillus thuringiensis toxin.

The heavy closed boxes represent the partially homologous regions, open for HD-73, dashed for HD-1 Dipel.

Fig. 4 - Shows the alignment of the two Bacillus thuringiensis DNA sequences HD-1 and HD-73 in order to give the greatest homology.

Vertical dashed lines indicate matches; boxes represent, for each recombinant obtained by the present method, regions wherein the recombination has occurred; numbers in bold above boxes represent the new recombinant obtained HY 6, HY 15, HY 107, HY 53.

DNA sequence nucleotides are indicated and numbered according to the original numeration given in the published DNA sequences HD-1 (Schnepf et al., 1985) and HD-73 (Adang et al., 1985).

Fig. 5 - Shows the optimal alignment of polypeptide deduced from the sequences of HD-1 Dipel and HD-73 genes.

PCT/EP90/01145

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 18, line 12 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

INSTITUT PASTEUR
COLLECTION NATIONAL DE MICRO-ORGANISMES

Address of depositary institution (including postal code and country) [4]

25, RUE DU DR. ROUX
75015 PARIS

Date of deposit [5]	Accession Number [6]
June 28, 1989	

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

AUSTRALIA
JAPAN
EUROPEAN PATENT
SOVIET UNION
UNITE STATES OF AMERICA

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

12. 07. 90

TON A.D. VELDEN
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

(January 1985)

In Panel A (modified after Geiser et al., 1986) is schematically represented the alignment of the entire polypeptides; a vertical line represents an unmatched residue, short bars near the horizontal lines are deletions.

Panel B represents the aminoacid sequences aligned for the region corresponding to the partial homology region, at the DNA sequence level, used for the recombination in pT173 and pGEM173.

The sequence indicated in this figure corresponds to the region underlined in Panel A.

An aminoacid residue identical in HD-1 Dipel and HD-73 or in the hybrid products is denoted by a bar.

- represents an aminoacid residue deleted to obtain maximum alignment.

Figs 6 a-e Show the DNA nucleotide sequences of hypervariable regions of hybrid Bacillus thuringiensis genes obtained by in vivo recombination according to the present invention.

The DNA sequence has been determined sperimentally on both DNA strands for all the recombinant hybrid genes obtained.

HD-1 DNA sequence is in bold letters. Numeration is as follows: Position 1 corresponds to nucleotide 1521 of the HD-1 DNA sequence (Schnepf, 1985) while the last nucleotide corresponds to nucleotide 2091 of the HD-73 DNA sequence (Adang et al., 1985).

CLAIMS

1. Hybrid DNA sequences characterized in that they are obtained by in vivo recombination of two genes coding for an insecticidal toxin protein.

2. Hybrid DNA sequences according to Claim 1, wherein said hybrid DNA sequences have been obtained by in vivo recombination of two Bacillus thuringiensis genes coding for the Bacillus thuringiensis crystal toxin protein.

3. Hybrid DNA sequences according to Claims 1-2, wherein said hybrid DNA sequences have been obtained by in vivo recombination of two Bacillus thuringiensis variety kurstaki genes coding for the Bacillus thuringiensis crystal toxin protein.

4. Hybrid DNA sequences according to Claims 1-3, wherein said DNA sequences have been obtained by in vivo recombination of the Bacillus thuringiensis variety kurstaki HD-1 Dipel gene and the Bacillus thuringiensis var. kurstaki HD-73 gene, both genes coding for the Bacillus thuringiensis crystal toxin protein.

5. Hybrid DNA sequences according to Claims 1-4 wherein said hybrid DNA sequences have been obtained by in vivo recombination of the hypervariable regions of the Bacillus thuringiensis var. kurstaki HD-1 Dipel gene and of Bacillus thuringiensis var. kurstaki HD-73 gene, both genes coding for the Bacillus thuringiensis crystal toxin protein.

6. Hybrid DNA sequence HY 5 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

LOCUS HY5 713 BP ENTERED 5/22/87

ORIGIN IN VIVO RECOMBINANT HD1/HD73 #5

```
   1 AATTCBCATT CCCTTTATTT BBGAATBCBG BBAATBCABC TCCACCCBTA CTTBTCTCAT
  61 TAACTBGTTT BGGBATTTTT ABAACATTAT CTTCACCTTT ATATABAAGA ATTATACTTB
 121 BTTCABGCCC AAATAATCAB BAACTBTTTB TCCTTBATBG AACBBABTTT TCTTTTBCCT
 181 CCCTAACBAC CAACTTBCCT TCCACTATAT ATABACAAAG BGBTACABTC BATTCACTAG
 241 ATBTAATACC BCCACABAAT AACAACGTGC CACCTAGGCA AGGATTTAGT CATCGATTAA
 301 GCCATGTTTC AATGTTTCGT TCAGGCTTTA GTAATAGTAG TGTAAGTATA ATAAGAGCTC
 361 CTATGTTCTC TTGGATACAT CGTAGTGCTG AATTTAATAA TATAATTGCA TCGGATAGTA
 421 TTACTCAAAT CCCTGCAGTG AAGGGAAACT TTCTTTTTAA TGGTTCTGTA ATTTCAGGAC
 481 CAGGATTTAC TGGTGGGGAC TTAGTTAGAT TAAATAGTAG TGGAAATAAC ATTCAGAATA
 541 GAGGGTATAT TGAAGTTCCA ATTCACTTCC CATCGACATC TACCAGATAT CGAGTTCGTG
 601 TACGGTATGC TTCTGTAACC CCGATTCACC TCAACGTTAA TTGGGGTAAT TCATCCATTT
 661 TTTCCAATAC AGTACCAGCT ACAGCTACGT CATTAGATAA TCTACAATCA AGT
```

7. Hybrid DNA sequence HY 45 according to Claims 1-5 characterized by an hypervariable region having the following sequence:

LOCUS HY45 707 BP ENTERED 5/23/89

ORIGIN IN VIVO RECOMBINANTS HD-1/HD73 #45,66

```
   1 AATTCBCATT CCCTTTATTT BGGAATBCBG BBAATBCABC TCCACCCBTA CTTBTCTCAT
  61 TAACTBGTTT BGGBATTTTT AGAACATTAT CTTCACCTTT ATATABAAGA ATTATACTTB
 121 BTTCABGCCC AAATAATCAB BAACTBTTTB TCCTTBATBG AACBBABTTT TCTTTTBCCT
 181 CCCTAACBAC CAACTTBCCT TCCACTATAT ATABACAAAB BBBTACABTC BATTCACTAB
 241 ATBTAATACC BCCACABBAT AATABTBTAC CACCTCBTBC BBBATTTABC CATCBATTBA
 301 BTCATBTTAC AATBCTBABC CAABCABCTB BABCABTTTA CACCTTBABA BCTCCAACBT
 361 TTTCTTBBAT ACATCBTABT BCTBAATTTA ATAATATAAT TBCATCBBAT ABTATTACTC
 421 AAATCCCTBC ABTBAABBBA AACTTTCTTT TTAATBBTTC TBTAATTTCA BBACCABBAT
 481 TTACTBBTBB BBACTTABTT ABATTAAATA BTABTBBAAA TAACATTCAB AATABABBBT
 541 ATATTBAABT TCCAATTCAC TTCCCATCBA CATCTACCAB ATATCBABTT CBTBTACBBT
 601 ATBCTTCTBT AACCCCBATT CACCTCAACB TTAATTBBBB TAATTCATCC ATTTTTTCCA
 661 ATACABTACC ABCTACABCT ACBTCATTAB ATAATCTACA ATCAABT
```

8. Hybrid DNA sequence HY 3 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

LOCUS HY3 710BP ENTERED 5/23/89

ORIGIN IN VIVO RECOMBINANTS HD-1/HD73 #3,4,7

```
  1 AATTCBCATT CCCTTTATTT BGGAATBCGG BGAATBCABC TCCACCCBTA CTTBTCTCAT
 61 TAACTGGTTT BGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTB
121 BTTCAGGCCC AAATAATCAB BAACTBTTTB TCCTTBATBG AACGGABTTT TCTTTTBCCT
181 CCCTAACGAC CAACTTBCCT TCCACTATAT ATAGACAAAG BGGTACABTC BATTCACTAG
241 ATBTAATACC BCCACAGGAT AATABTBTAC CACCTCBTBC BGGATTTABC CATCBATTBA
301 BTCATGTTAC AATBCTBAGC CAAGCAGCTB GAGCABTTTA CACCTTBAGA BCTCCAACBT
361 TTTCTTBGCA BCATCBCAGT BCTBAATTTA ATAATATAAT TCCTTCATCA CAAATTACAC
421 AAATACCTTT AACAAAATCT ACTAATCTTB BCTCTBGAAC TTCTBTCBTT AAAGGACCAG
481 BATTTACTGG TGGGGACTTA GTTAGATTAA ATAGTAGTGG AAATAACATT CAGAATAGAG
541 GGTATATTGA AGTTCCAATT CACTTCCCAT CGACATCTAC CAGATATCGA GTTCGTGTAC
601 GGTATGCTTC TGTAACCCCG ATTCACCTCA ACGTTAATTG GGGTAATTCA TCCATTTTTT
661 CCAATACAGT ACCAGCTACA GCTACGTCAT TAGATAATCT ACAATCAAGT
```

9. Hybrid DNA sequence HY 21 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

LOCUS HY21 713BP ENTERED 5/23/89

ORIGIN IN VIVO RECOMBINANT HD-1/HD73 #21

```
  1 AATTCBCATT CCCTTTATTT BGBAATBCBB BBAATBCABC TCCACCCBTA CTTBTCTCAT
 61 TAACTGGTTT BGGGATTTTT AGAACATTAT CTTCACCTTT ATATABAAGA ATTATACTTB
121 BTTCABBCCC AAATAATCAB BAACTBTTTB TCCTTBATBB AACBBABTTT TCTTTTBCCT
181 CCCTAACGAC CAACTTBCCT TCCACTATAT ATABACAAAG BGGTACABTC BATTCACTAG
241 ATBTAATACC BCCACAGGAT AATABTBTAC CACCTCBTBC BGGATTTABC CATCBATTBA
301 BCCATGTTTC AATGTTTCGT TCAGGCTTTA GTAATAGTAG TGTAAGTATA ATAAGAGCTC
361 CTATGTTCTC TTGGATACAT CGTAGTGCTG AATTTAATAA TATAATTGCA TCGGATAGTA
421 TTACTCAAAT CCCTGCAGTG AAGGGAAACT TTCTTTTTAA TGGTTCTGTA ATTTCAGGAC
481 CAGGATTTAC TGGTGGGGAC TTAGTTAGAT TAAATAGTAG TGGAAATAAC ATTCAGAATA
541 GAGGGTATAT TGAAGTTCCA ATTCACTTCC CATCGACATC TACCAGATAT CGAGTTCGTG
601 TACGGTATGC TTCTGTAACC CCGATTCACC TCAACGTTAA TTGGGGTAAT TCATCCATTT
661 TTTCCAATAC AGTACCAGCT ACAGCTACGT CATTAGATAA TCTACAATCA AGT
```

10. Hybrid DNA sequence HY 32 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

LOCUS HY32 707BP ENTERED 5/23/89

ORIGIN IN VIVO RECOMBINANT HD-1/HD73 #32

```
  1 AATTCBCATT CCCTTTATTT BBBAATBCBB BBAATBCABC TCCACCCBTA CTTBTCTCAT
 61 TAACTBBTTT BBBBATTTTT ABAACATTAT CTTCACCTTT ATATABAABA ATTATACTTB
121 BTTCABBCCC AAATAATCAB BAACTBTTTB TCCTTBATBB AACBBABTTT TCTTTTBCCT
181 CCCTAACBAC CAACTTBCCT TCCACTATAT ATABACAAAB BBBTACABTC BATTCACTAB
241 ATBTAATACC BCCACABBAT AATABTBTAC CACCTCBTBC BBBATTTABC CATCBATTBA
301 BTCATBTTAC AATBCTBABC CAABCABCTB BABCABTTTA CACCTTBABA BCTCCTATBT
361 TCTCTTBBAT ACATCBTABT BCTBAATTTA ATAATATAAT TBCATCBBAT ABTATTACTC
421 AAATCCCTBC ABTBAABBBA AACTTTCTTT TTAATBBTTC TBTAATTTCA BBACCABBAT
481 TTACTBBTBB BBACTTABTT ABATTAAATA BTABTBBAAA TAACATTCAB AATABABBBT
541 ATATTBAABT TCCAATTCAC TTCCCATCBA CATCTACCAB ATATCBABTT CBTBTACBBT
601 ATBCTTCTBT AACCCCBATT CACCTCAACB TTAATTBBBB TAATTCATCC ATTTTTTCCA
661 ATACABTACC ABCTACABCT ACBTCATTAB ATAATCTACA ATCAABT
```

11. Hybrid DNA sequence HY 6 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

LOCUS HT6 710BP UPDATED 5/23/89

ORIGIN IN VIVO RECOMBINANT HD-1/HD73 #6

```
  1 AATTCBCATT CCCTTTATTT BBBAATBCBB BAAATBCABC TCCACAACAA CBTATTBTTB
 61 CTCAACTABB TCABBBCBTB TATABAACAT TATCBTCCAC TTTATATABA ABACCTTTTA
121 ATATABBBAT AAATAATCAA CAACTATCTB TTCTTBACBB BACABAATTT BCTTATBBAA
181 CCTCCTCAAA TTTBCCATCC BCTBTATACA BAAAAABCBB AACBBTABAT TCBCTBBATB
241 AAATACCBCC ACABAATAAC AACBTBCCAC CTABBCAABB ATTTABTCAT CBATTAABCC
301 ATBTTTCAAT BTTTCBTTCA BBCTTTABTA ATABTABTBT AABTATAATA ABABCTCCTA
361 TBTTCTCTTB BATACATCBT ABTBCTBAAT TTAATAATAT AATTBCATCB BATABTATTA
421 CTCAAATCCC TBCABTBAAB BBAAACTTTC TTTTTAATBB TTCTBTAATT TCABBACCAB
481 BATTTACTBB TBBBBACTTA BTTABATTAA ATABTABTBB AAATAACATT CABAATABAB
541 BBTATATTBA ABTTCCAATT CACTTCCCAT CBACATCTAC CABATATCBA BTTCBTBTAC
601 BBTATBCTTC TBTAACCCCB ATTCACCTCA ACBTTAATTB BBBTAATTCA TCCATTTTTT
661 CCAATACABT ACCABCTACA BCTACBTCAT TABATAATCT ACAATCAABT
```

12. Hybrid DNA sequence HY 53 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

LOCUS HY53 710BP UPDATED 5/22/89

ORIGIN IN VIVO RECOMBINANTS HD-1/HD73 #53,64,107

```
   1 AATTCBCATT CCCTTTTATTT BBBAATBCBB BBAATBCABC TCCACAACAA CGTATTGTTG
  61 CTCAACTAGG TCAGGGCGTG TATAGAACAT TATCGTCCAC TTTATATAGA AGACCTTTTA
 121 ATATAGGGAT AAATAATCAA CAACTATCTG TTCTTGACGG GACAGAATTT GCTTATGGAA
 181 CCTCCTCAAA TTTGCCATCC GCTGTATACA GAAAAAGCGG AACGGTAGAT TCGCTGGATG
 241 AAATACCGCC ACAGAATAAC AACGTGCCAC CTAGGCAAGG ATTTAGTCAT CGATTAAGCC
 301 ATGTTTCAAT GTTTCGTTCA GGCTTTAGTA ATAGTAGTGT AAGTATAATA AGAGCTCCTA
 361 TGTTCTCTTG GATACATCGT AGTGCTGAAT TTAATAATAT AATTGCATCG GATAGTATTA
 421 CTCAAATCCC TGCAGTGAAG GGAAACTTTC TTTTTAATGG TTCTGTAATT TCAGGACCAG
 481 GATTTACTGG TGGGGACTTA GTTAGATTAA ATAGTAGTGG AAATAACATT CAGAATAGAG
 541 GGTATATTGA AGTTCCAATT CACTTCCCAT CGACATCTAC CAGATATCGA GTTCGTGTAC
 601 GGTATGCTTC TGTAACCCCG ATTCACCTCA ACGTTAATTG GGGTAATTCA TCCATTTTTT
 661 CCAATACAGT ACCAGCTACA GCTACGTCAT TAGATAATCT ACAATCAAGT
```

13. Hybrid DNA sequence HY 2 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

LOCUS HY2 713BP UPDATED 5/23/89

ORIGIN IN VIVO RECOMBINANT HD-1/HD73 #2

```
   1 AATTCBCATT CCCTTTTATTT BGBAATBCBB BBAATBCABC TCCACCCBTA CTTBTCTCAT
  61 TAACTBGTTT BGGGATTTTT AGAACATTAT CTTCACCTTT ATATABAABA ATTATACTTB
 121 BTTCABBCCC AAATAATCAB BAACTBTTTB TCCTTBATBB AACBGABTTT TCTTTTBCCT
 181 CCCTAACGAC CAACTTBCCT TCCACTATAT ATAGACAAAG BGGTACABTC BATTCACTAB
 241 ATBTAATACC BCCACAGGAT AATAGTBTGC CACCTAGGCA AGGATTTAGT CATCGATTAA
 301 GCCATGTTTC AATGTTTCGT TCAGGCTTTA GTAATAGTAG TGTAAGTATA ATAAGAGCTC
 361 CTATGTTCTC TTGGATACAT CGTAGTGCTG AATTTAATAA TATAATTGCA TCGGATAGTA
 421 TTACTCAAAT CCCTGCAGTG AAGGGAAACT TTCTTTTTAA TGGTTCTGTA ATTTCAGGAC
 481 CAGGATTTAC TGGTGGGGAC TTAGTTAGAT TAAATAGTAG TGGAAATAAC ATTCAGAATA
 541 GAGGGTATAT TGAAGTTCCA ATTCACTTCC CATCGACATC TACCAGATAT CGAGTTCGTG
 601 TACGGTATGC TTCTGTAACC CCGATTCACC TCAACGTTAA TTGGGGTAAT TCATCCATTT
 661 TTTCCAATAC AGTACCAGCT ACAGCTACGT CATTAGATAA TCTACAATCA AGT
```

14. Hybrid DNA sequence HY 127 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

LOCUS HY127 713BP ENTERED 5/23/89

ORIGIN IN VIVO RECOMBINANT HD-1/HD73 #127

```
   1 AATTCBCATT CCCTTTATTT BBBAATBCBB BBAATBCABC TCCACCCBTA CTTBTCTCAT
  61 TAACTBGTTT BBBGATTTTT ABAACATTAT CTTCACCTTT ATATABAABA ATTATACTTB
 121 BTTCABBCCC AAATAATCAB BAACTBTTTB TCCTTBATBB AACBBABTTT TCTTTTBCCT
 181 CCCTAACBAC CAACTTBCCT TCCACTATAT ATABACAAAB BBGTACAGTC BATTCACTAB
 241 ATBTAATACC BCCACABBAT AATAGTBTAC CACCTCBTBC BBGATTTABT CATCBATTAA
 301 BCCATBTTTC AATBTTTCBT TCABBCTTTA BTAATAGTAB TBTAABTATA ATAABABCTC
 361 CTATBTTCTC TTBBATACAT CBTAGTBCTB AATTTAATAA TATAATTBCA TCBBATAGTA
 421 TTACTCAAAT CCCTBCABTB AABBBAAACT TTCTTTTTAA TBBTTCTBTA ATTTCABBAC
 481 CABBATTTAC TBBTBBBBAC TTAGTTAGAT TAAATAGTAB TBBAAATAAC ATTCABAATA
 541 BABBBTATAT TBAABTTCCA ATTCACTTCC CATCBACATC TACCABATAT CBABTTCBTB
 601 TACBBTATBC TTCTBTAACC CCBATTCACC TCAACBTTAA TTBBBBTAAT TCATCCATTT
 661 TTTCCAATAC ABTACCABCT ACABCTACBT CATTABATAA TCTACAATCA ABT
```

15. Hybrid DNA sequence HY 126 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

LOCUS HY126 707 BP ENTERED 5/22/89

ORIGIN IN VIVO RECOMBINANT HD-1/HD 73 #126

```
   1 AATTCBCATT CCCTTTATTT BBBAATBCBB BBAATBCABC TCCACCCBTA CTTBTCTCAT
  61 TAACTBGTTT BBBGATTTTT ABAACATTAT CTTCACCTTT ATATABAABA CCTTTTAATA
 121 TABBBATAAA TAATCAACAA CTATCTBTTC TTBACBBBAC ABAATTTBCT TATBBAACCT
 181 CCTCAAATTT BCCATCCBCT BTATACABAA AAABCBBAAC BBTABATTCB CTBBATBAAA
 241 TACCBCCACA BAATAACAAC BTBCCACCTA BBCAABBATT TABTCATCBA TTAABCCATB
 301 TTTCAATBTT TCBTTCABBC TTTABTAATA BTABTBTAAB TATAATAABA BCTCCTATBT
 361 TCTCTTBBAT ACATCBTABT BCTBAATTTA ATAATATAAT TBCATCBBAT ABTATTACTC
 421 AAATCCCTBC ABTBAABBBA AACTTTCTTT TTAATBBTTC TBTAATTTCA BBACCABBAT
 481 TTACTBBTBB BBACTTABTT ABATTAAATA BTABTBBAAA TAACATTCAB AATABABBBT
 541 ATATTBAABT TCCAATTCAC TTCCCATCBA CATCTACCAB ATATCBABTT CBTBTACBBT
 601 ATBCTTCTBT AACCCCBATT CACCTCAACB TTAATTBBBB TAATTCATCC ATTTTTTCCA
 661 ATACABTACC ABCTACABCT ACBTCATTAB ATAATCTACA ATCAABT
```

16. Hybrid insecticidal crystal toxin protein encoded by the hybrid DNA sequences according to Claims 1-15.

17. Hybrid insecticidal crystal toxin proteins having substantially the immunological properties of the hybrid insecticidal crystal toxin protein

according to Claim 16.

18. Muteins of the hybrid insecticidal crystal toxin proteins according either to Claim 16 or Claim 17 wherein said muteins have been obtained by standardized genetic engineering techniques such as site-specific mutagenesis, random mutagenesis, site-specific glycosilation, and their activities are reconducible to the insecticidal activities of their parental hybrid insecticidal crystal toxin proteins according to either Claim 16 or Claim 17.

19. Plasmid vectors characterized in that they contain two genes coding for an insecticidal toxin protein and having enough residual homology to be able to recombine in vivo, wherein said genes upon in vivo recombination produce the hybrid DNA sequences of Claims 1-15.

20. Plasmid vectors according to Claim 19 further characterized in that the genes coding for the insecticidal toxin protein are accociated and separated on said plasmid vectors by a DNA fragment acting as an antibiotic resistance marker.

21. Plasmid vectors according to Claim 19 wherein the genes coding for an insecticidal toxin protein are Bacillus thuringiensis genes coding for the Bacillus thuringiensis crystal toxin protein.

22. Plasmid vectors according to Claim 21 wherein the Bacillus thuringiensis genes are Bacillus thuringiensis variety kurstaki genes coding for the Bacillus thuringiensis variety kurstaki crystal toxin protein.

23. Plasmid vectors according to Claim 22 wherein the Bacillus thuringiensis variety kurstaki

genes have been derived from Bacillus thuringiensis variety kurstaki HD-1 Dipel and from Bacillus thuringiensis variety kurstaki HD 73, said genes coding both for crystal toxin protein.

24. Plasmid vectors according to Claim 23 wherein said plasmid vectors are pT173 and pGEM173.

25. Plasmid vector characterized in that it contains a first gene coding for a first insecticidal toxin protein and a second gene coding for a second insecticidal toxin protein, having enough amology in order to be able to recombine in vivo, so that it produces hybrid DNA sequences coding for a third insecticidal toxin protein, wherein said first gene is characteristic of the hypervariable region of a first bacterial strain, while the said second gene is characteristic of the hypervariable region of a second bacterial strain substantially different from the said first strain.

26. Vector according to claim 25 characterized in that said first bacterial strain and said second bacterial strain belong to the same genera, preferably Bacillus type.

27. Vector according to claim 26 characterized in that said first bacterial strain and said second bacterial strain belong to the same species, preferably Bacillus thuringiensis type.

28. Vector according to claim 27 characterized in that said first bacterial strain and said second bacterial strain belong to the same subspecies, preferably of the Bacillus thuringiensis kurstaki type.

29. Plasmid expression vectors characterized in

that they have been obtained by in vivo recombination of the two genes coding for an insecticidal toxin protein contained in plasmid vectors according to Claims 19 and 25 wherein said plasmid vectors obtained by in vivo recombination contain the hybrid DNA sequences of Claims 1-15.

30. Plasmid expression vectors containing the hybrid DNA sequences according to Claims 1-15 under the control of expression regulatory functions.

31. Plasmid expression vectors according to Claim 29 wherein said expression regulatory functions include, but are not limited to, the lac system, the Trp system, the major operator and promoter regions of phage λ, the tac system, the β-lac system.

32. Plasmid expression vectors containing the hybrid DNA sequences according to Claims 1-15 wherein said plasmid expression vectors are used to transform plant cells.

33. Plant cells transformed by plasmid expression vectors according to Claim 29.

34. Transgenic insect resistant plants containing the hybrid DNA sequences according to Claims 1-15.

35. Genetically engineered plant colonizing microorganism containing the hybrid DNA sequences according to Claim 1-15.

36. Host cells transformed by the plasmid expression vectors according to Claim 29.

37. Host cells according to Claim 36 wherein said host cells are various strains of Bacillus, including B. subtilis, B. thuringiensis, yeasts, Agrobacterium, baculoviruses, Rhizobium.

38. Host cells according to Claim 36 wherein said host cells are Escherichia Coli host cells.

39. Host cells according to Claim 38 wherein said host cells can be recombinant unproficient cells rec A^-.

40. Escherichia coli host cells transformed with plasmid vector pT173.

41. Escherichia coli host cells transformed with plasmid vector pGEM 173.

42. A process for preparing the hybrid DNA sequences of Claims 1-15 by in vivo recombination of two or more genes coding for an insecticidal toxin protein and having enough residual homology to be able to recombine in vivo.

43. A process according to Claim 42, wherein said genes coding for an insecticidal protein are Bacillus thuringiensis genes coding for the Bacillus thuringiensis crystal toxin protein.

44. Pesticidal compositions and formulations containing the hybrid crystal toxin proteins of Claims 16 and 17 in combination with suitable eccipients, diluents, fillers, aggregant and the like.

45. Use of the hybrid crystal toxin proteins of Claim 17 to control and to combat insect pest.

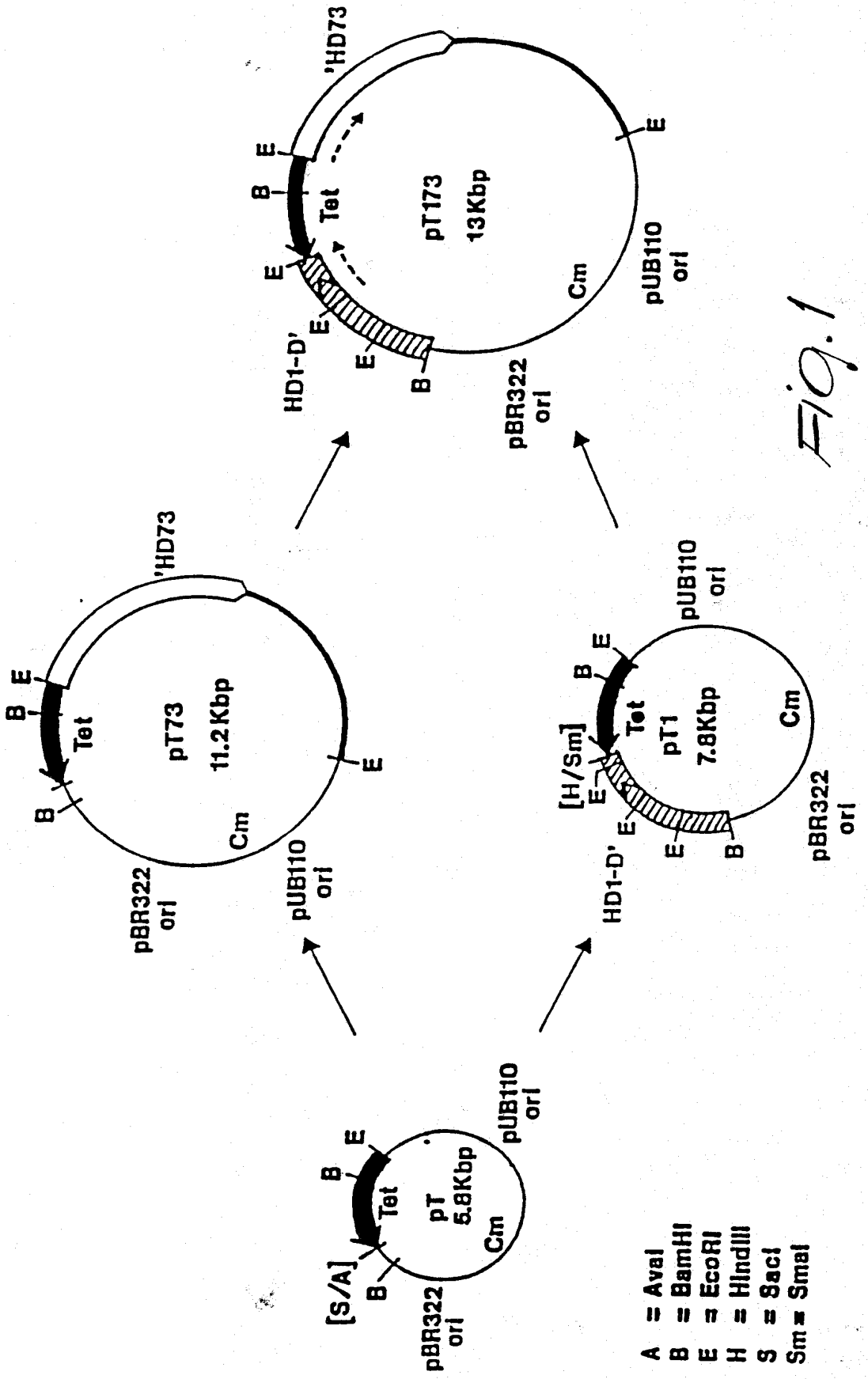

'HD73
pT173
13Kbp
Tet
Cm
pUB110 ori
pBR322 ori
HD1-D'
pT73
11.2Kbp
pT1
7.8Kbp
[H/Sm]
pT
5.8Kbp
[S/A]
B
E
A = AvaI
B = BamHI
E = EcoRI
H = HindIII
S = SacI
Sm = SmaI

Fig. 1

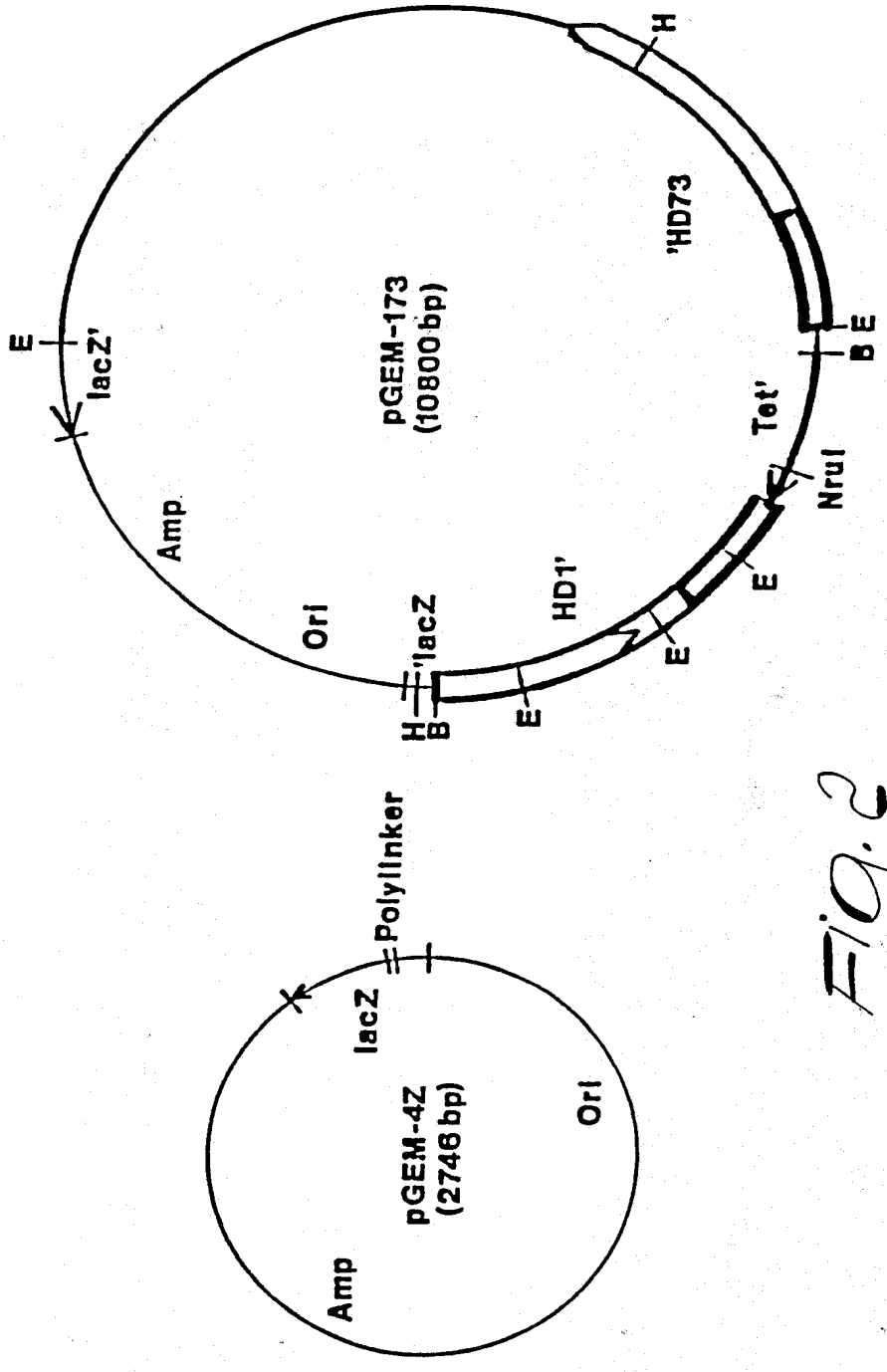

pGEM-173
(10800 bp)
Amp
Ori
lacZ'
E
'lacZ
H
B
HD1'
E
E
Nrul
Tet'
B E
'HD73
H
pGEM-4Z
(2748 bp)
Amp
Ori
lacZ
Polylinker

Fig. 2

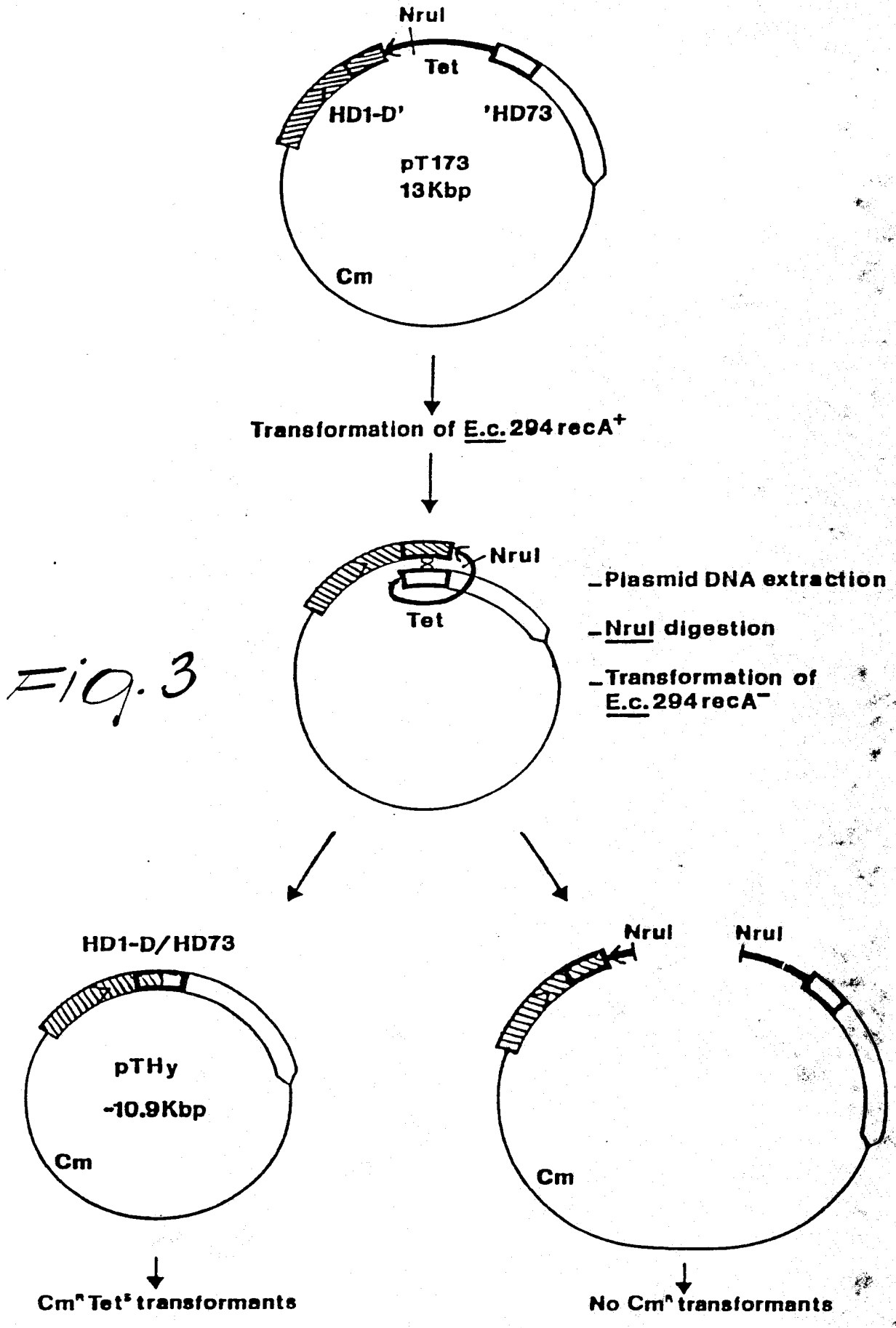
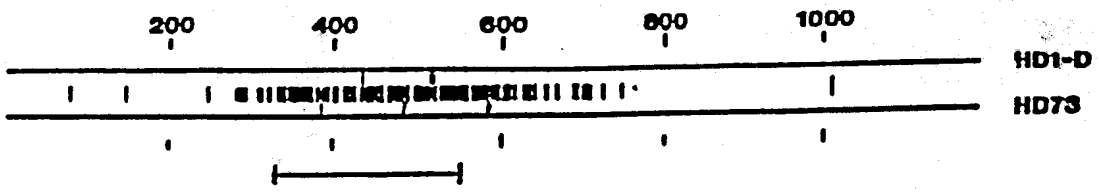

NruI
Tet
HD1-D'
'HD73
pT173
13Kbp
Cm
Transformation of E.c. 294 recA+
NruI
Tet
_Plasmid DNA extraction
_NruI digestion
_Transformation of E.c. 294 recA−
HD1-D/HD73
pTHy
~10.9Kbp
Cm
NruI
NruI
Cm
Cmr Tets transformants
No Cmr transformants

Fig. 3

```
HD 1 Dipel gene                                          LIMITS:  1521
HD 73 gene                                               LIMITS:  1383

                                          6.15   64.53.107
1521    AATTCGCATTCCCTTTATTTGGGAATGCGGGGAATGCAGCTCCACCCGTACTTGT  CTC
        |||| | || ||  ||| ||| | |  ||| |||||||||||||     || | |   |
1383     ATTCACTTTTCCGCTATATGGAACTATGGGAAATGCAGCTCCACAACAACGTATTGTTG
                                                  126
1579    ATTAACT GGTTTGGGGATTTTTAGAACATTATCTTCACCTTTATATAGAAGAATTATAC
        | |||| ||| |||  | | ||||||||||||| ||  ||||||||||||||  | |
1442    CTCAACTAGGTCAGGGCGTGTATAGAACATTATCGTCCACTTTATATAGAAGACCT T

1638    TTGGTTCAGGCCCAAATAATCAGGAACTGTTTGTCCTTGATGGAACGGAGTTTTCTTTTG
        ||  |  ||  |||||||||  |||| | ||| ||||| || || || ||| ||| ||
1499    TTAATATAGGGATAAATAATCAACAACTATCTGTTCTTGACGGGACAGAATTTGCTTATG

1698    CCTCCCTAACGACCAACTTGCCTTCCACTATATATAGACAAAGGGGTACAGTCGATTCAC
            |||  |  | || ||||| ||| || |||| ||| |||| || || || ||||| |
1559     GAACCT  CCTCAAATTTGCCATCCGCTGTATACAGAAAAAGCGGAACGGTAGATTCGC
                    5                    2                  127
1758    TAGATGTAATACCGCCACAGGATAATAGTGTACCACCTCGTGCGGGATTTAGCCATCGAT
        | |||| |||||||||||||  |||| |  || |||||| |     |||||||| |||||||
1616    TGGATGAAATACCGCCACAGAATAACAACGTGCCACCTAGGCAAGGATTTAGTCATCGAT

                21                                                32
1818    TGAGTCATGTTACAATGCTGAG CCAAGC   AGCTGGAGCAGTTTA  CACCTTGAGAG
        | |||  |||||| ||||| |  |  ||  ||    ||  ||| ||  |   | |||||
1676    TAAGCCATGTTTCAATGTTTCGTTCAGGCTTTAGTAATAGTAGTGTAAGTATAATAAGAG
                    66.45
1872    CTCCAACGTTTTCTTGGCAGCATCGCAGTGCTGAATTTAATAATATAATTCCTTCATCAC
        ||||  | |||  |||||||   |||||  |||||||||||||||||||||||| | ||
1736    CTCCTATGTTCTCTTGGATACATCGTAGTGCTGAATTTAATAATATAATTGCATCGGATA

1932    AAATTACACAAATACCTTTAACAAAATCTACTAATCTTGGCTCTGGAACTTCTGTCGTTA
          ||||| ||||| |||  |   ||    |   ||||     |||    ||||||  ||
1796    GTATTACTCAAATCCCTGCAGTGAAGGGAAACTTTCTTTTTAATGG   TTCTGTAATTT
                3.4.7
1992    AAGGACCAGGATTTACAGGAGGAGATATTCTTCGAAGAACTTCACCTGGCCAGA  TTTC
        ||||||||||||||||  || || ||  |  ||  ||  ||  |     |||  | |  |||
1853    CAGGACCAGGATTTACTGGTGGGGACTTAGTTAGATTAAATAGTAGTGGAAATAACATTC

2050    AACCTTAAGAGTAAAT  A  TT  ACTGCA    CCAT   TATCACAAAGATATCGGG
        |   |  || ||| ||  |  ||  | | ||    ||||    |||    |||||||| |
1913    AGAATAGAGGGTATATTGAAGTTCCAATTCACTTCCCATCGACATCTACCAGATATCGAG

2097    TAAGAATTCGCTACGCTTCTACTACAAATTTACAATTCCATACATCAATTGACGGAA  G
        |  |  | || || ||||||   ||     | ||  | ||  || | |||||  | ||
1973    TTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCT CA ACGTTAATTGGGGTAATTC

2155    ACCTATTAATCAGGGTAATTTTTCAGCAAC TATGAGTAGTGGGAGTAATTTACAGTCCG
        | | |||  |    ||  |  |||| ||  |  ||  |  || |||| |||| ||
2031    ATCCATTTTTTCCAATACAGTACCAGCTACAGCTACGTCATTAGA TAATCTACAATCAA

2214    GA
        |
2090    GT

Matches = 456     Mismatches = 226     Unmatched = 40
Length = 722      Matches/length =  63.2 percent
```

Fig. 4



B

HYBRID PROTEINS FROM RECOMBINANT GENES

```
HD1-D       333  FAFPLFSNAGNAAPPV^LVSLTSLSIFRTLSSPLYRRIILSSGPNNQELFVLDGTEFSFAS
HY7,3,4          ------------------------------------------------------------
HY66,45          ------------------------------------------------------------
HY32             ------------------------------------------------------------
HY127,21         ------------------------------------------------------------
HY2              ------------------------------------------------------------
HY5              ------------------------------------------------------------
HY126            ----------------------------------P^PNI-I---Q-S-------AYGT
HY6,53,64,107    ----------QQRI-AQL-Q-VY-----T----P^PNI-I---Q-S-------AYGT
HY15             ----------KCSSTTTYCCSTRSGRV*
HD73             -T---Y-TM------QQRI-AQL-Q-VY------T----P^FNI-I---Q-S-------AYGT

HD1-D       393  LTTNLPSTIYRQRGTVDSLDVIPPQDNSVPPRAGFSHRLSHVTM^LSQA^AGAVYTLRAPT
HY7,3,4          ------------------------------------------------------------
HY66,45          ------------------------------------------------------------
HY32             ------------------------------------------------------------M
HY127,21         ----------------------------------------S-FR-GFSNSS-SII---M
HY2              ----------------------------------Q---------S-FR-GFSNSS-SII---M
HY5              --------------------------N-N----Q---------S-FR-GFSNSS-SII---M
HY126            ^SS----AV--KS-------E----N-N----Q---------S-FR-GFSNSS-SII---M
HY6,53,64,107    ^SS----AV--KS-------E----N-N----Q---------S-FR-GFSNSS-SII---M
HD73             ^SS----AV--KS-------E----N-N----Q---------S-FR-GFSNSS-SII---M

HD1-D       452  FSWQHRSAEFNNIIPSSQITQIPLTKSTNLSSGTSVVKGPGFTGGDILRRTSPGQISTLRV
HY7,3,4          ----------------------------------------------LV-LN-S-NNIQN-G
HY66,45          ---I----------A-DS-----AV-GNFLFN-^--IS--------LV-LN-S-NNIQN-G
HY32             ---I----------A-DS-----AV-GNFLFN-^--IS--------LV-LN-S-NNIQN-G
HY127,21         ---I----------A-DS-----AV-GNFLFN-^--IS--------LV-LN-S-NNIQN-G
HY2              ---I----------A-DS-----AV-GNFLFN-^--IS--------LV-LN-S-NNIQN-G
HY5              ---I----------A-DS-----AV-GNFLFN-^--IS--------LV-LN-S-NNIQN-G
HY126            ---I----------A-DS-----AV-GNFLFN-^--IS--------LV-LN-S-NNIQN-G
HY6,53,64,107    ---I----------A-DS-----AV-GNFLFN-^--IS--------LV-LN-S-NNIQN-G
HD73             ---I----------A-DS-----AV-GNFLFN-^--IS--------LV-LN-S-NNIQN-G

HD1-D       513  NITAPLSQRYRV^RIRYASTTNLQFHTSIDGRPINQGNF^SATMSSGSNLQSGS 564
HY7,3,4          Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
HY66,45          Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
HY32             Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
HY127,21         Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
HY2              Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
HY5              Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
HY126            Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
HY6,53,64,107    Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
HD73             Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
```

Fig. 5

```
LOCUS       HY45          707 BP                          ENTERED  5/23/89
ORIGIN      IN VIVO RECOMBINANTS HD-1/HD73 #45,66
        1 AATTCBCATT CCCTTTATTT BBBAATBCBB BBAATBCABC TCCACCCBTA CTTBTCTCAT
       61 TAACTBBTTT BBBBATTTTT ABAACATTAT CTTCACCTTT ATATABAABA ATTATACTTB
      121 BTTCABBCCC AAATAATCAB BAACTBTTTB TCCTTBATBB AACBBABTTT TCTTTTBCCT
      181 CCCTAACBAC CAACTTBCCT TCCACTATAT ATABACAAAB BBBTACABTC BATTCACTAB
      241 ATBTAATACC BCCACABBAT AATABTBTAC CACCTCBTBC BBBATTTABC CATCBATTBA
      301 BTCATBTTAC AATBCTBABC CAABCABCTB BABCABTTTA CACCTTBABA BCTCCAACBT
      361 TTTCTTBBAT ACATCBTAGT GCTBAATTTA ATAATATAAT TBCATCBBAT AGTATTACTC
      421 AAATCCCTBC AGTBAABBBA AACTTTCTTT TTAATBGTTC TBTAATTTCA BBACCAGBAT
      481 TTACTBGTBB BBACTTAGTT AGATTAAATA BTAGTBBAAA TAACATTCAB AATABABBBT
      541 ATATTBAAGT TCCAATTCAC TTCCCATCBA CATCTACCAB ATATCBAGTT CBTBTACBBT
      601 ATBCTTCTBT AACCCCBATT CACCTCAACB TTAATTBBBB TAATTCATCC ATTTTTTCCA
      661 ATACABTACC ABCTACABCT ACBTCATTAB ATAATCTACA ATCAAGT
//

LOCUS       HY3           710 BP                          ENTERED  5/23/89
ORIGIN      IN VIVO RECOMBINANTS HD-1/HD73 #3,4,7
        1 AATTCBCATT CCCTTTATTT BBBAATBCBB BBAATBCABC TCCACCCBTA CTTBTCTCAT
       61 TAACTBBTTT BBBBATTTTT ABAACATTAT CTTCACCTTT ATATABAABA ATTATACTTB
      121 BTTCABBCCC AAATAATCAB BAACTBTTTB TCCTTBATBB AACBBABTTT TCTTTTBCCT
      181 CCCTAACBAC CAACTTBCCT TCCACTATAT ATABACAAAB BBBTACABTC BATTCACTAB
      241 ATBTAATACC BCCACABBAT AATABTBTAC CACCTCBTBC BBBATTTABC CATCBATTBA
      301 BTCATBTTAC AATBCTBABC CAABCABCTB BABCABTTTA CACCTTBABA BCTCCAACBT
      361 TTTCTTBBCA BBATCBCABT BCTBAATTTA ATAATATAAT TCCTTCATCA CAAATTACAC
      421 AAATACCTTT AACAAAATCT ACTAATCTTB BCTCTBBAAC TTCTBTCBTT AAABBACCAB
      481 BATTTACTBB TBBBBACTTA BTTABATTAA ATABTABTBB AAATAACATT CABAATABAB
      541 BBTATATTBA ABTTCCAATT CACTTCCCAT CBACATCTAC CABATATCBA BTTCBTBTAC
      601 BBTATBCTTC TBTAACCCCB ATTCACCTCA ACBTTAATTB BBBTAATTCA TCCATTTTTT
      661 CCAATACABT ACCABCTACA BCTACBTCAT TABATAATCT ACAATCAAGT
//
```

Fig. 6a

```
LOCUS       HY6             710 BP                          UPDATED  5/23/89
ORIGIN      IN VIVO RECOMBINANT HD-1/HD73 #6
        1 AATTCBCATT CCCTTTATTT BBBAATBCBB BAAATGCAGC TCCACAACAA CGTATTGTTG
       61 CTCAACTAGG TCAGGGCGTG TATAGAACAT TATCGTCCAC TTTATATAGA AGACCTTTTA
      121 ATATAGGGAT AAATAATCAA CAACTATCTG TTCTTGACGG GACAGAATTT GCTTATGGAA
      181 CCTCCTCAAA TTTGCCATCC GCTGTATACA GAAAAAGCGG AACGGTAGAT TCGCTGGATG
      241 AAATACCGCC ACAGAATAAC AACGTGCCAC CTAGGCAAGG ATTTAGTCAT CGATTAAGCC
      301 ATGTTTCAAT GTTTCGTTCA GGCTTTAGTA ATAGTAGTGT AAGTATAATA AGAGCTCCTA
      361 TGTTCTCTTG GATACATCGT AGTGCTGAAT TTAATAATAT AATTGCATCG GATAGTATTA
      421 CTCAAATCCC TGCAGTGAAG GGAAACTTTC TTTTTAATGG TTCTGTAATT TCAGGACCAG
      481 GATTTACTGG TGGGGACTTA GTTAGATTAA ATAGTAGTGG AAATAACATT CAGAATAGAG
      541 GGTATATTGA AGTTCCAATT CACTTCCCAT CGACATCTAC CAGATATCGA GTTCGTGTAC
      601 GGTATGCTTC TGTAACCCCG ATTCACCTCA ACGTTAATTG GGGTAATTCA TCCATTTTTT
      661 CCAATACAGT ACCAGCTACA GCTACGTCAT TAGATAATCT ACAATCAAGT
//

LOCUS       HY53            710 BP                          UPDATED  5/22/89
ORIGIN      IN VIVO RECOMBINANTS HD-1/HD73 #53,64,107
        1 AATTCBCATT CCCTTTATTT BBBAATBCBB BBAATBCABC TCCACAACAA CGTATTGTTG
       61 CTCAACTAGG TCAGGGCGTG TATAGAACAT TATCGTCCAC TTTATATAGA AGACCTTTTA
      121 ATATAGGGAT AAATAATCAA CAACTATCTG TTCTTGACGG GACAGAATTT GCTTATGGAA
      181 CCTCCTCAAA TTTGCCATCC GCTGTATACA GAAAAAGCGG AACGGTAGAT TCGCTGGATG
      241 AAATACCGCC ACAGAATAAC AACGTGCCAC CTAGGCAAGG ATTTAGTCAT CGATTAAGCC
      301 ATGTTTCAAT GTTTCGTTCA GGCTTTAGTA ATAGTAGTGT AAGTATAATA AGAGCTCCTA
      361 TGTTCTCTTG GATACATCGT AGTGCTGAAT TTAATAATAT AATTGCATCG GATAGTATTA
      421 CTCAAATCCC TGCAGTGAAG GGAAACTTTC TTTTTAATGG TTCTGTAATT TCAGGACCAG
      481 GATTTACTGG TGGGGACTTA GTTAGATTAA ATAGTAGTGG AAATAACATT CAGAATAGAG
      541 GGTATATTGA AGTTCCAATT CACTTCCCAT CGACATCTAC CAGATATCGA GTTCGTGTAC
      601 GGTATGCTTC TGTAACCCCG ATTCACCTCA ACGTTAATTG GGGTAATTCA TCCATTTTTT
      661 CCAATACAGT ACCAGCTACA GCTACGTCAT TAGATAATCT ACAATCAAGT
//
```

Fig. 6b

```
LOCUS       HY21          713 BP                          ENTERED  5/23/89
ORIGIN      IN VIVO RECOMBINANT HD-1/HD73 #21
        1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
       61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTG
      121 GTTCAGGCCC AAATAATCAG GAACTGTTTG TCCTTGATGG AACGGAGTTT TCTTTTGCCT
      181 CCCTAACGAC CAACTTGCCT TCCACTATAT ATAGACAAAG GGGTACAGTC GATTCACTAG
      241 ATGTAATACC GCCACAGGAT AATAGTGTAC CACCTCGTGC GGGATTTAGC CATCGATTGA
      301 GCCATGTTTC AATGTTTCGT TCAGGCTTTA GTAATAGTAG TGTAAGTATA ATAAGAGCTC
      361 CTATGTTCTC TTGGATACAT CGTAGTGCTG AATTTAATAA TATAATTGCA TCGGATAGTA
      421 TTACTCAAAT CCCTGCAGTG AAGGGAAACT TTCTTTTTAA TGGTTCTGTA ATTTCAGGAC
      481 CAGGATTTAC TGGTGGGGAC TTAGTTAGAT TAAATAGTAG TGGAAATAAC ATTCAGAATA
      541 GAGGGTATAT TGAAGTTCCA ATTCACTTCC CATCGACATC TACCAGATAT CGAGTTCGTG
      601 TACGGTATGC TTCTGTAACC CCGATTCACC TCAACGTTAA TTGGGGTAAT TCATCCATTT
      661 TTTCCAATAC AGTACCAGCT ACAGCTACGT CATTAGATAA TCTACAATCA AGT
//

LOCUS       HY32          707 BP                          ENTERED  5/23/89
ORIGIN      IN VIVO RECOMBINANT HD-1/HD73 #32
        1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
       61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTG
      121 GTTCAGGCCC AAATAATCAG GAACTGTTTG TCCTTGATGG AACGGAGTTT TCTTTTGCCT
      181 CCCTAACGAC CAACTTGCCT TCCACTATAT ATAGACAAAG GGGTACAGTC GATTCACTAG
      241 ATGTAATACC GCCACAGGAT AATAGTGTAC CACCTCGTGC GGGATTTAGC CATCGATTGA
      301 GTCATGTTAC AATGCTGAGC CAAGCAGCTG GAGCAGTTTA CACCTTGAGA GCTCCTATGT
      361 TCTCTTGGAT ACATCGTAGT GCTGAATTTA ATAATATAAT TGCATCGGAT AGTATTACTC
      421 AAATCCCTGC AGTGAAGGGA AACTTTCTTT TTAATGGTTC TGTAATTTCA GGACCAGGAT
      481 TTACTGGTGG GGACTTAGTT AGATTAAATA GTAGTGGAAA TAACATTCAG AATAGAGGGT
      541 ATATTGAAGT TCCAATTCAC TTCCCATCGA CATCTACCAG ATATCGAGTT CGTGTACGGT
      601 ATGCTTCTGT AACCCCGATT CACCTCAACG TTAATTGGGG TAATTCATCC ATTTTTTCCA
      661 ATACAGTACC AGCTACAGCT ACGTCATTAG ATAATCTACA ATCAAGT
//
```

Fig. 6c

```
LOCUS       HY126          707 BP                          ENTERED  5/22/89
ORIGIN      IN VIVO RECOMBINANT HD-1/HD73 #126
        1 AATTCBCATT CCCTTTATTT BBBAATBCBB BBAATBCABC TCCACCCBTA CTTBTCTCAT
       61 TAACTBBTTT BBBBATTTTT ABAACATTAT CTTCACCTTT ATATABAABA CCTTTTAATA
      121 TAGGGATAAA TAATCAACAA CTATCTGTTC TTGACGGGAC AGAATTTGCT TATGGAACCT
      181 CCTCAAATTT GCCATCCGCT GTATACAGAA AAAGCGGAAC GGTAGATTCG CTGGATGAAA
      241 TACCGCCACA GAATAACAAC GTGCCACCTA GGCAAGGATT TAGTCATCGA TTAAGCCATG
      301 TTTCAATGTT TCGTTCAGGC TTTAGTAATA GTAGTGTAAG TATAATAAGA GCTCCTATGT
      361 TCTCTTGGAT ACATCGTAGT GCTGAATTTA ATAATATAAT TGCATCGGAT AGTATTACTC
      421 AAATCCCTGC AGTGAAGGGA AACTTTCTTT TTAATGGTTC TGTAATTTCA GGACCAGGAT
      481 TTACTGGTGG GGACTTAGTT AGATTAAATA GTAGTGGAAA TAACATTCAG AATAGAGGGT
      541 ATATTGAAGT TCCAATTCAC TTCCCATCGA CATCTACCAG ATATCGAGTT CGTGTACGGT
      601 ATGCTTCTGT AACCCCGATT CACCTCAACG TTAATTGGGG TAATTCATCC ATTTTTTCCA
      661 ATACAGTACC AGCTACAGCT ACGTCATTAG ATAATCTACA ATCAAGT
//

LOCUS       HY5            713 BP                          ENTERED  5/22/89
ORIGIN      IN VIVO RECOMBINANT HD1/HD73 #5
        1 AATTCBCATT CCCTTTATTT BBBAATBCBB BBAATBCABC TCCACCCBTA CTTBTCTCAT
       61 TAACTBBTTT BBBBATTTTT ABAACATTAT CTTCACCTTT ATATABAABA ATTATACTTB
      121 BTTCABBCCC AAATAATCAB BAACTBTTTB TCCTTBATBB AACBBABTTT TCTTTTBCCT
      181 CCCTAACBAC CAACTTBCCT TCCACTATAT ATABACAAAB BBBTACABTC BATTCACTAB
      241 ATBTAATACC BCCACABAAT AACAACGTGC CACCTAGGCA AGGATTTAGT CATCGATTAA
      301 GCCATGTTTC AATGTTTCGT TCAGGCTTTA GTAATAGTAG TGTAAGTATA ATAAGAGCTC
      361 CTATGTTCTC TTGGATACAT CGTAGTGCTG AATTTAATAA TATAATTGCA TCGGATAGTA
      421 TTACTCAAAT CCCTGCAGTG AAGGGAAACT TTCTTTTTAA TGGTTCTGTA ATTTCAGGAC
      481 CAGGATTTAC TGGTGGGGAC TTAGTTAGAT TAAATAGTAG TGGAAATAAC ATTCAGAATA
      541 GAGGGTATAT TGAAGTTCCA ATTCACTTCC CATCGACATC TACCAGATAT CGAGTTCGTG
      601 TACGGTATGC TTCTGTAACC CCGATTCACC TCAACGTTAA TTGGGGTAAT TCATCCATTT
      661 TTTCCAATAC AGTACCAGCT ACAGCTACGT CATTAGATAA TCTACAATCA AGT
//
```

FIG. 6d

```
LOCUS       HY2            713 BP                          UPDATED  5/23/89
ORIGIN      IN VIVO RECOMBINANT HD-1/HD73 #2
        1 AATTCBCATT CCCTTTATTT BBBAATBCBB BBAATBCABC TCCACCCBTA CTTBTCTCAT
       61 TAACTBBTTT BBBBATTTTT ABAACATTAT CTTCACCTTT ATATABAABA ATTATACTTB
      121 BTTCABBCCC AAATAATCAB BAACTBTTTB TCCTTBATBB AACBBABTTT TCTTTTBCCT
      181 CCCTAACBAC CAACTTBCCT TCCACTATAT ATABACAAAB BBBTACABTC BATTCACTAB
      241 ATBTAATACC BCCACABBAT AATABTBTBC CACCTAGGCA AGGATTTAGT CATCGATTAA
      301 GCCATGTTTC AATGTTTCGT TCAGGCTTTA GTAATAGTAG TGTAAGTATA ATAAGAGCTC
      361 CTATGTTCTC TTGGATACAT CGTAGTGCTG AATTTAATAA TATAATTGCA TCGGATAGTA
      421 TTACTCAAAT CCCTGCAGTG AAGGGAAACT TTCTTTTTAA TGGTTCTGTA ATTTCAGGAC
      481 CAGGATTTAC TGGTGGGGAC TTAGTTAGAT TAAATAGTAG TGGAAATAAC ATTCAGAATA
      541 GAGGGTATAT TGAAGTTCCA ATTCACTTCC CATCGACATC TACCAGATAT CGAGTTCGTG
      601 TACGGTATGC TTCTGTAACC CCGATTCACC TCAACGTTAA TTGGGGTAAT TCATCCATTT
      661 TTTCCAATAC AGTACCAGCT ACAGCTACGT CATTAGATAA TCTACAATCA AGT
/

LOCUS       HY127          713 BP                          ENTERED  5/23/89
ORIGIN      IN VIVO RECOMBINANT HD-1/HD73 #127
        1 AATTCBCATT CCCTTTATTT BBBAATBCBB BBAATBCABC TCCACCCBTA CTTBTCTCAT
       61 TAACTBBTTT BBBBATTTTT ABAACATTAT CTTCACCTTT ATATABAABA ATTATACTTB
      121 BTTCABBCCC AAATAATCAB BAACTBTTTB TCCTTBATBB AACBBABTTT TCTTTTBCCT
      181 CCCTAACBAC CAACTTBCCT TCCACTATAT ATABACAAAB BBBTACABTC BATTCACTAB
      241 ATBTAATACC BCCACABBAT AATABTBTAC CACCTCBTBC BBBATTTABT CATCGATTAA
      301 GCCATGTTTC AATGTTTCGT TCAGGCTTTA GTAATAGTAG TGTAAGTATA ATAAGAGCTC
      361 CTATGTTCTC TTGGATACAT CGTAGTGCTG AATTTAATAA TATAATTGCA TCGGATAGTA
      421 TTACTCAAAT CCCTGCAGTG AAGGGAAACT TTCTTTTTAA TGGTTCTGTA ATTTCAGGAC
      481 CAGGATTTAC TGGTGGGGAC TTAGTTAGAT TAAATAGTAG TGGAAATAAC ATTCAGAATA
      541 GAGGGTATAT TGAAGTTCCA ATTCACTTCC CATCGACATC TACCAGATAT CGAGTTCGTG
      601 TACGGTATGC TTCTGTAACC CCGATTCACC TCAACGTTAA TTGGGGTAAT TCATCCATTT
      661 TTTCCAATAC AGTACCAGCT ACAGCTACGT CATTAGATAA TCTACAATCA AGT
/
```

Fig. 6e

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 90/01145

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A01N63/02 ; C12N15/32 ; C12P21/02 ; //C12N15/62

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	EP,A,141484 (BIOGEN N.V.) see page 8, line 18 - page 11, line 10; figures 1, 4 see page 12, line 22 - page 13, line 12 (cited in the application) ---	1-5, 18-19, 21-23, 25-39 42-45
Y	EP,A,228838 (MYCOGEN CORP.) see page 2, lines 15 - 22; claims see page 7; examples 3-5 (cited in the application) ---	1-5, 18-19, 21-23, 25-39 42-45

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19 OCTOBER 1990	30. 10. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	ANDRES S.M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, June 1989, WASHINGTON US pages 4037 - 4041; GE, A.Z. et al.: "Location of the Bombyx mori specificity domain on a Bacillus thuringiensis delta-endotoxin protein" see the whole document	1-5, 18-19, 21-23, 25-39
	---	42-45
Y	NUCLEIC ACIDS RESEARCH. vol. 11, no. 16, 1983, ARLINGTON, VIRGINIA pages 5661 - 5669; WEBER,H. & WEISSMANN,C.: "Formation of genes coding for hybrid proteins by recombination between related, cloned genes in E.coli" see the whole document (cited in the application)	1-5, 18-19, 21-23, 25-39
	---	42-45

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9001145
SA 38941

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 17/10/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-141484	15-05-85	JP-A- 60070083	20-04-85
EP-A-228838	15-07-87	JP-A- 62143689	26-06-87